human lens proteins:
aspects of aging.

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human lens proteins: aspects of aging.

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„experience is not what happens to a man, but what a man does with what has happened to him".

A. Huxley

aan wijlen mijn vader, aan mijn moeder en aan mijn vrienden

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CHAPTER I

INTRODUCTION

Both the ancient Egyptians and Hindus seem to have known eye surgeons; however, the first scientific approaches date from the father of medicine Hippocrates (406 - 377 B.C.) and the Roman Celsus in the first century. They considered the lens to be a drop of liquid, originating from the brain; in this fluid sight was assumed to be located and cataract was considered to be a condition in which a thick flowing was present in front of the lens.

This belief of Celsus has influenced medicine for ages and it was not until the philosophy of the late Renaissance that not only were the old - ever dominating - religious rules forbidding experiments on dead bodies questioned, but also concern with sciences increased tremendously. This resulted in a proper anatomical description of the lens, although its function was understood only as production of a clear image on the retina (Kepler, 1604) and the retina was then believed to have the photoreceptor role. However, it was another 150 years until Daviel first published a description of a cataractous lens extraction.

Berzelius[1] was the first one to speak of the "lens crystallina", as he found it to be a protein containing body and named the protein "krystallin". This substance was believed to be similar to serum albumin.

Mörner[2] separated the lens protein into several fractions, one of which was water-insoluble and was called "albumoid". The water-soluble proteins were divided in three classes: α- and β-crystallin and an albumin fraction. He also described distinct differences between cortex and nucleus as to their contents of water-soluble and water-insoluble constituents.

Jess[3] had already noted in 1922 that structural changes of the water-soluble proteins might be involved in the formation of

albumoid, which could be a cause for cataract. Burky and Woods[4] were in 1928 the first to use the name γ-crystallin instead of albumin for the third water-soluble component.

The physiological function of the lens is the formation of a clear (inverted) image on the retina. Since the first experimental proof by von Helmholtz, little has changed in the accomodation theory. The lens is connected with the ciliary muscle around its entire circumference through the zonulae ciliares; contraction and relaxation of this muscle is responsible for accomodation. This allows the lens to take different shapes in accord with its elastic properties. Von Helmholtz's theory of vision necessitates that the lens remains a passive body during accomodation.

However, Gullstrand[5] gave evidence for both extracapsular and intracapsular factors involved in accomodation. Kleifeld et al.[6,7,8] confirmed these ideas by showing that during accomodation a decrease of certain metabolic compounds is found. Their work suggested the involvement of an active intracapsular system in addition to the presence of a primitive contractile mechanism in lens fibers. Klethi and Mandel[9] related ATP content with visual accomodation in different species. Rafferty and Goossens hypothesized on the function of cytoplasmic filaments both in maintaining the spherical shape of the lens and in providing a contractile mechanism. Finally, Kibbelaar et al.[11] provided this hypothesis with a model for the *in vivo* organization of actin in the eye lens of accomodating species.

As to the function of the lens, it is increasingly evident that the old von Helmholtz theory on accomodation is not complete any longer and that more mechanisms are involved than only the extracapsular traction and relaxation by the ciliary muscle.

In the adult state the lens is a spherically shaped, avascular organ,surrounded by a strong capsule. The nutrients necessary for the cell metabolism are obtained from the aqueous,

which flows around the lens and is permanently renewed. Before birth, till 5 months of gestation, the lens is surrounded by a dense web of vessels (see Fig. 1).

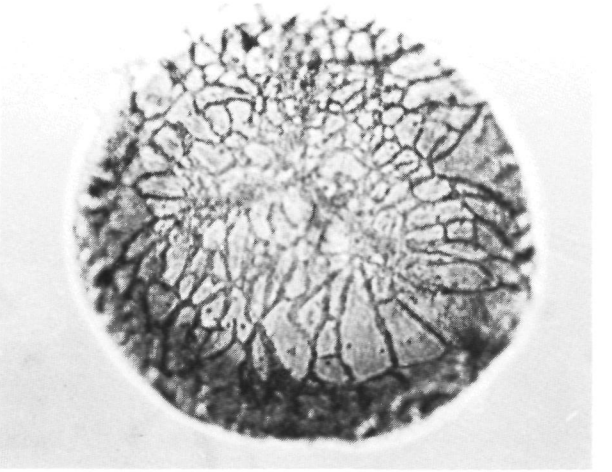

Fig. 1. Human fetal lens, 8 weeks of gestation; posterior surface with vascular network.

A section through a lens of an 8 week-old fetus is shown in Fig. 2. The unique growth pattern is understandable from this picture. The epithelial cells on the anterior surface undergo mitosis near the equator; then they migrate from a pre-elongation zone to the elongation zone. During this elongation the surface of the cell increases enormously[12] and so the synthesis of plasma membranes also has to augment. Eventually, most of the cell organelles in the area now called the lens fiber cell are lost, including the nucleus; this terminal differentiation is reminiscent of the differentiation of the erythrocyte. However, protein synthesis still continues in the lens fiber cell[13], which reflects the presence of stabilized poly-

ribosomes[14,15].

Each new layer of cells is formed at the periphery of the lens and laid down on the outside of the already present fibers. As the lens lacks the ability to shed cells, this means that within the inner lens those fibers are present which were formed during embryogenesis.

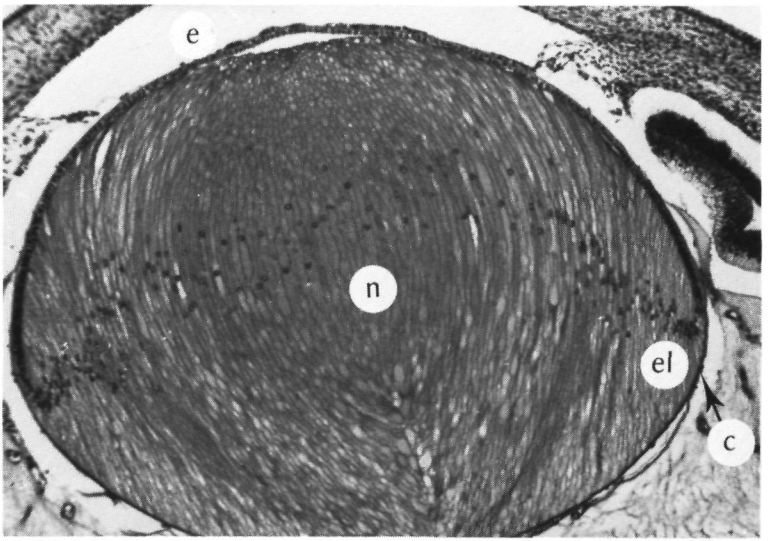

Fig. 2. Human fetal lens, 8 weeks of gestation; transverse section, showing the unique growth pattern of the eye lens. e = epithelium; el = elongation zone; c = capsule; n = nucleus.

In this respect the eye lens is very unique: no other tissue is known to conserve its life history by simply storing all cells ever formed in a regular growth pattern. Formation of cells goes on throughout life, although, with aging, at a slower rate. The closely packed cells are connected by many intercellular junctions, as has been shown by Benedetti et al.[16,17] and by Goodenough[18].

Synthetic activity eventually stops in the nucleus of the lens[19,20].

Why the lens is so attractive for research can be summarized as follows:

a. the lens gives fewer isolation problems than many other tissues, as there are only lens cells present.
b. the growth pattern makes it possible to study post-translational events, such as degradation, deamidation, aggregation and insolubilization.
c. the lens contains a relatively high percentage of membranes.
d. the lens is very suitable for the isolation of mRNA[21] and the preparation of a viable cell-free system[22], thus facilitating studies at the molecular level.

Bovine lenses are by far the most intensively investigated and much information has been obtained on this species; e.g., primary gene products[13], post-translational modifications[23], aging[24], quaternary structure[25] and cytoskeleton[16,17,26]. In addition, there has recently been great progress made at the molecular biological level using chick lens[27,28]. Human lenses have been studied in respect to aging and the development of cataract. An excellent review on mammalian lens has been written by Harding and Dilley[29]. However, for understandable reasons, little is known about the very young and fetal human lens. It is presumed that human lens is not very much different from the bovine lens.

In Chapter II we will show some of the changes, as seen on SDS-PAGE, that occur between fetal life and 65 years of age in the water-soluble and water-insoluble portions of the lens; we will compare these phenomena to the situation in cattle. This investigation was extended by separation of lens proteins of different ages by means of column chromatography (Chapter III).

Chapter IV deals with the *in vitro* biosynthesis of proteins in

intact fetal human lenses.

In addition, we attempted to isolate polysomes from fetal lenses and to translate them in a reticulocyte cell-free system. This will be described in Chapter V.

There have been several attempts to culture human lens epithelium[30,31,32,33,34] with rather controversial results; this has been more successful for many other species. Results of our investigations are given in Chapter VI.

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CHAPTER II

EFFECT OF AGING ON THE WATER-SOLUBLE AND WATER-INSOLUBLE PROTEIN PATTERN IN NORMAL HUMAN LENS.

INTRODUCTION.

Interest in the water-soluble proteins from normal human lens has increased considerably in recent years. In particular, studies on their synthesis, post-translational changes, aggregation, insolubilization characteristics, aging and their role in cataractogenesis have been undertaken (Clark, Zigman and Lerman, 1969; Roy and Spector, 1976; Kabasawa, Barber and Kinoshita, 1977; Bushell and Duncan, 1978; Jedziniak, Baram and Chylack, 1978; Kramps, De Jong, Wollensak and Hoenders, 1978; Ringens, Liem-The, Hoenders and Wollensak, 1978; Sandberg and Closs, 1978a and 1978b; Garner and Spector, 1979; Zigler, Horwitz and Kinoshita, 1980). Whereas for a number of species the soluble crystallins have been investigated in young lenses, this is hitherto not the case for humans. Therefore, little information is available concerning the proteins in young human lens and virtually nothing is known of how many of them represent primary gene products. As there is no protein synthesis in the nucleus (Dilley and Van Heyningen, 1976) the lens is extremely appropriate for aging studies. This is particularly interesting for human lens.

Usually the findings in the human lens are compared with those of other mammals, especially cattle.

For the water-insoluble part of the lens the situation is very similar. Broekhuyse (1969) reported an analysis of the lipid composition of the human lens fiber membranes and age-related changes (1973). A similar study on the proteins of these membranes has recently been published by Alcalá, Valentine and Maisel (1980). The latter authors used a considerable number of young lenses. Also other groups conducted studies on the human lens and its proteins (Roy and Spector, 1978a and 1978b; Garner, Garner and Spector, 1979; Horwitz, Robertson, Wong, Zigler and Kinoshita, 1979; Roy, Spector and Farnsworth, 1979; Horwitz and Wong, 1980; Zigler,Horwitz and Kinoshita, 1981).

It is the aim of the present communication to give a survey of age-related changes in the polypeptide patterns of the water-soluble

and water-insoluble proteins from cortex and nucleus of normal human lenses.

MATERIALS AND METHODS.

Lenses were decapsulated and, except for the fetal human lens, the cortex was separated from the nucleus. In all cases the ratio between cortex and nucleus was approximately 2 : 1. Nuclei of the adult lenses were cut into 6 - 8 pieces. Samples were gently stirred in 1.5 ml of a fresh buffer, containing 20 mM Tris-HCl, 80 mM NaCl, 1 mM EDTA and 10 mM DTE at 4^{o} C for 60 min. These homogenates were centrifuged at 10,000 x g for 20 min. The supernatant fraction was recovered and frozen. The pellet was resuspended according to the description above and the procedure was repeated. As we intended to isolate a pure water-soluble fraction mechanical homogenization was avoided. Both supernatant fractions were pooled, TCA was added to a final concentration of 5 %, samples were centrifuged at 5000 x g for 10 min and finally lyophilized.

The water-insoluble pellets, obtained after the second centrifugation at 10,000 x g, were washed 3 times in 20 ml of the same buffer. A_{280} of the supernatant fraction was less than 0.05 after the third washing. This procedure leaves open the possibility of slight contamination of the water-insoluble fraction with water-soluble components.

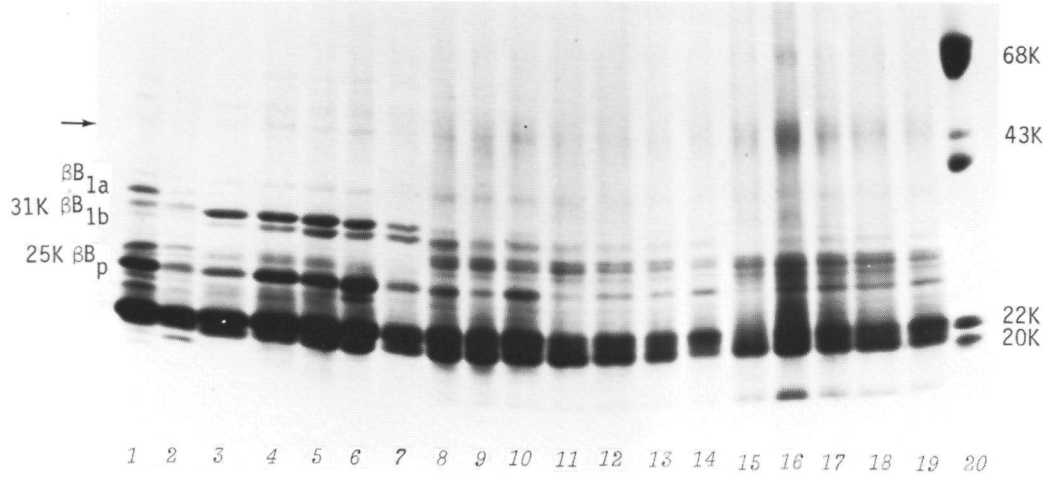
Samples were dissolved in a solution of 0.0625 M Tris-HCl, pH 7.8, 2 % SDS, 0.1 % DTE, 10 % sucrose, 0.002 % Bromophenol Blue, to a final concentration of approximately 1 mg/ml and incubated at 37^{o} C for 3 hours.

Electrophoresis was performed according to a modification of the original procedure described by Laemmli (1970), using a gradient slab gel system (7 - 18 %). Bovine serum albumin, ovalbumin and calf α-crystallin were used as markers.

RESULTS AND DISCUSSION.

A typical SDS-PAGE pattern of the water-soluble human lens proteins is shown in Fig. 1a. The following conclusions can be drawn: from 14 years onward (lanes 8 - 19) there are no obvious differences between the patterns of the cortical and nuclear proteins. Dilley and Harding (1975) claimed that the neonatal pattern has the greatest complexity and the sharpness of the electrophoretic separation deteriorates with increasing age. Our results are in agreement with the latter statement, but not with the former: with increasing age, a number of minor bands become visible in the range between 18,000 and 28,000 daltons, with a relatively high amount of polypeptide chains in the 20 Kdalton region. Dilley and Harding (1975) provided evidence for significant deamidation of α-crystallin before birth and possibly during infancy. Sandberg and Closs (1978a and 1978b) demonstrated the heterogeneous nature of old human lens crystallin fractions. It would not be surprising if, during infancy, degradation processes also take place, which might account for the greater complexity with age. However, it remains questionable whether these are all post-translational products derived from crystallins.

In all samples from individuals older than 14 years a polypeptide chain of approximately 10 Kdaltons is found (Fig. 1a, lanes 8 - 19). A polypeptide of nearly the same size has been described by Jedziniak et al. (1978). These authors considered it to be unique for human lens; however, we found it also in low concentrations in calf nucleus (see Fig. 1a, lane 2) but not yet in 6 month-old human lens (lanes 6 and 7), which is in agreement with results of Zigler et al. (1981). Roy and Spector (1978b) have shown that it is derived from the αA chain. Garner and Spector (1978) and Garner et al. (1979) also considered this 10 Kdalton polypeptide to be a result of post-translational degradation; they found that it contained components from different populations of polypeptides, including crystallins.



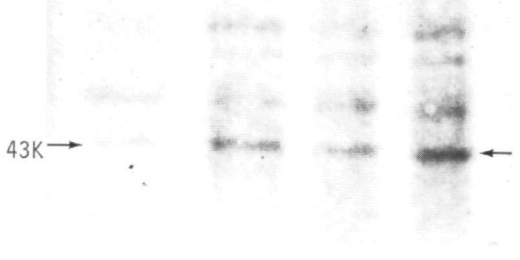


Fig. 1a: SDS-PAGE pattern of the water-soluble proteins from human lens fibers. (C=cortex; N=nucleus). The amount of protein applied to each lane was approximately 15 µg.
1. calf C *2*. calf N *3*. human fetus, 23rd week of gestation *4*. 3 days C *5*. 3 days N *6*. 6 months C *7*. 6 months N *8*. 14 years C *9*. 14 years N *10*. 20 years C *11*. 20 years N *12*. 30 years C *13*. 30 years N *14*. 45 years C *15*. 45 years N *16*. 55 years C *17*. 55 years N *18*. 65 years C *19*. 65 years N *20*. markers.

Fig. 1b: Detail from SDS-PAGE pattern shown in Fig. 1a. The arrow indicates the 43 Kdalton polypeptide. (lanes 3 - 6 from Fig. 1a).

This 10 Kdalton constituent has been reported to appear gradually during the period between 10 and 40 years of age. There is also some evidence that its occurrence may be due to degradation of already insolubilized material (Garner and Spector, 1978; Masters, Bada and Zigler, 1978).

We find a 43 Kdalton polypeptide in very low amounts in fetal and newborn lenses as a discrete band (Fig. 1a, lanes 3 - 6, arrow; Fig. 1b, arrow), which upon aging becomes more diffuse. This is in contrast to Garner and Spector's (1979) conclusion that the 43 Kdalton protein is not present in very young human lenses.

An interesting phenomenon is the gradual appearance with aging of a polypeptide of approximately 29 Kdaltons, concomitant with a relative decrease of a 29.5 Kdalton polypeptide (Fig. 1a, lanes 3 - 7). Whereas in fetal lens the 29.5 Kdalton band is the most prominent of these two polypeptides, the amount of the 29 Kdalton product increases in the newborn cortex and is even more enhanced in the newborn nucleus (lanes 4 and 5). In the 6 month-old lens this is more pronounced (lanes 6 and 7). This suggests that the 29 Kdalton band is a post-translational product derived from the 29.5 Kdalton band comparable with the situation in calf lens where a transition from βB_{1a} to βB_{1b} has been observed (Vermorken, Herbrink and Bloemendal, 1977). Apparently, in human lenses these polypeptides are faster migrating than in calf lenses (compare Fig. 1a, lanes 1 and 2 with lanes 3 - 7). In older lenses nothing of the 29.5 Kdalton and only a trace amount of the 29 Kdalton polypeptide remains.

In young human lens there is in addition to the 29 Kdalton band a major β-polypeptide visible, comigrating with calf βB_p. Its synthesis seems to be very high in the 6 month-old cortex (lane 6), is less pronounced in 14 and 20 year-old cortices (lanes 8 and 10), and is even less after 20 years of age.

The SDS-PAGE pattern obtained from the water-insoluble fractions is shown in Fig. 2.

Again the difference in sharpness between the bands from young

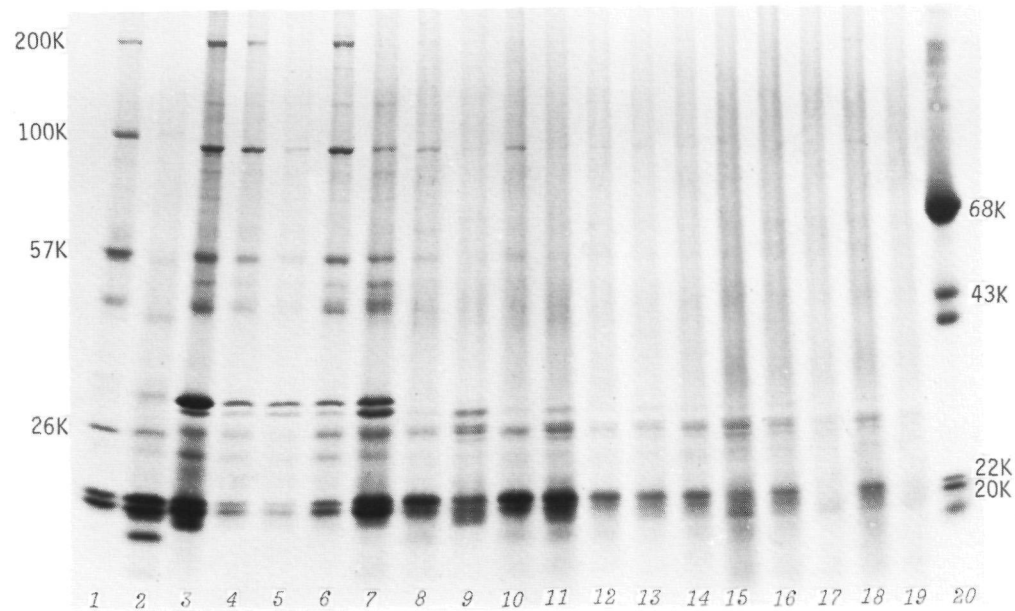


Fig. 2: SDS-PAGE patterns of the water-insoluble proteins from human lens fibers. (C=cortex; N=nucleus). The amount of protein applied to each lane was approximately 15 µg.
1. calf C *2*. calf N *3*. human fetus, 23rd week of gestation *4*. 3 days C *5*. 3 days N *6*. 6 months C *7*. 6 months N *8*. 14 years C *9*. 14 years N *10*. 20 years C *11*. 20 years N *12*. 30 years C *13*. 30 years N *14*. 45 years C *15*. 45 years N *16*. 55 years C *17*. 55 years N *18*. 65 years C *19*. 65 years N *20*. markers.

(up to 6 months) and old (14 years and older) lens fibers is striking. The high amount of the 29.5 Kdalton polypeptide in the fetal lens is very impressive (Fig. 2, lane 3), as is its gradual decrease towards the 6 month-old nucleus (lane 7) and its complete disappearance in the older cortices and nuclei. As in the water-soluble fraction, concomitant with this decrease there is an increase in the 29 Kdalton polypeptide, which can still be observed in minor amounts in the nuclei of 14 and 20 year-old lenses.

Alcalá et al. (1980) report on a transition of a 27 K polypeptide to a 25 K polypeptide. The authors believe that the 27 Kdalton component is comparable to the 26 Kdalton main intrinsic polypeptide in chick and calf. In all fractions we find a band in the 27 Kdalton region in some preparations concomitant with the 25 Kdalton component.

α-Crystallin polypeptides can be seen in all fractions, albeit the bands become more diffuse after 30 years (lanes 12 - 19). This is in agreement with findings by Alcalá et al. (1980). We are also able to show that there are some polypeptide chains with a molecular weight lower than 20 Kdaltons, which has been described earlier by Roy and Spector (1978a), by Garner et al. (1979) and by Alcalá et al. (1980); these authors reported molecular weights varying between 10 and 12 Kdaltons and their amount is thought to increase with aging. This is in concert with our present findings.

Very interesting are the bands in the young human fiber cells in the high molecular weight region; we have been able to perceive four bands with molecular weights of 200, 100, 57 and 43 Kdaltons, comigrating with calf myosin, α-actinin, vimentin and actin, respectively (though the human α-actinin moves slightly faster). Most of these cytoskeletal proteins have disappeared almost completely from lenses of 14 years and older (lanes 8 - 19). Only the band, comigrating with calf α-actinin is still visible in very low amounts in older lenses. It is fascinating to speculate about the role of the cytoskeleton in aging and in the maintenance of lens transparency. Actin has been proved to be present in the mammalian lens by Kibbelaar et al. (1979) and a possible role of the cytoskeleton in maintaining lens trans-

parency has earlier been suggested by Ireland, Maisel and Bradley (1978), by Mousa and Trevithick (1979), by Mousa, Creighton and Trevithick (1979) and by Bradley, Ireland and Maisel (1979). Such a role has recently also been proposed for human lens by Farnsworth et al. (1980) studying microtubules in human lens.

From our observations it appears that both in water-soluble and water-insoluble preparations, many "age"-dependent changes have already taken place in a 14 year-old human lens. We believe it is not sensible to take relative ages into account when comparing human lenses with those of other, shorter-living species. The lens has rather an absolute age: age-dependent, post-translational processes are absolute events and do not only occur after a certain period of biological aging. Our results clearly show that a typical aged protein pattern is not correlated with diminished transparency of the lens. Conversely, the aging of the lens starts at least at 14 years of age as far as the protein pattern is concerned.

Therefore we believe that in comparing 20 year-old lenses with 80 year-old lenses, one only compares one (already aged) sample with another (even more aged) sample. The distribution ratio between the individual crystallins upon aging will be dealt with in the following chapter.

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CHAPTER III.

PROTEIN DISTRIBUTION AND CHARACTERIZATION IN THE PRENATAL AND POSTNATAL HUMAN LENS.

INTRODUCTION.

It is well-documented that the vertebrate eye lens is a very convenient tissue for studying a number of fundamental biological processes. The lens contains a high amount of protein, remaining during the whole lifespan. Lens proteins cannot only serve as markers for terminal cell differentiation (Papaconstantinou, 1967; Bloemendal, 1979), but are also suitable for studies of the post-translational processes.

In contrast to cattle and chick lenses, relatively little work has been done on human lenses, with exception of some investigations on structural proteins in aging and cataract (Dilley and Harding, 1975; Roy and Spector, 1976; Coghlan and Augusteyn, 1977; Kabasawa, Barber and Kinoshita, 1977; Jedziniak, Baram and Chylack, 1978; Ringens, Liem-The, Hoenders and Wollensak, 1978; Garner and Spector, 1979; Zigler, Horwitz and Kinoshita, 1981).

The present chapter deals with:

1) The protein distribution in the developing and aging human lens, with special emphasis on the prenatal and postnatal stage of development.
2) The sedimentation analysis of these proteins and their subunit composition by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis and isoelectric focusing.
3) The determination of the N-terminal amino acid(s) of the low molecular weight fraction.
4) The question whether γ-crystallin is still present in the aged human eye lens, as has been suggested previously by Croft (1973) and by Ringens et al. (1978).

MATERIALS AND METHODS.

Preparation and separation of lens proteins. Lenses of six different age groups were taken: a) 76 years of age b) 40 years c) 14 years d) 3 days e) fetal human lenses between the 16th and 30th

week of gestation and f) fetal human lenses between the 8th and 16th week of gestation. All lenses were decapsulated and the adolescent (14 years) and adult lenses were separated in cortex and nucleus (cortex/nucleus = 2 : 1). The nuclei were cut into 6 - 8 pieces.

Samples were gently stirred in 2.0 ml of a freshly prepared buffer containing 20 mM Tris-HCl, 80 mM NaCl, 1 mM EDTA and 10 mM DTE, pH 7.3 at 4^{o} C for 240 min. The homogenates were centrifuged at 10,000 x g for 20 min. In order to improve the separation (Bloemendal and Zweers, 1976) the supernatant fraction was centrifuged at 78,000 x g at 4^{o} C for 16 hours. The pellet was resuspended in 2.0 ml of the original buffer and fractionated on a Biogel A-5m column (100 x 0.6 cm). Proportions of fractions were calculated by measuring the peak areas of the elution patterns.

Prior to analyses the protein samples were dialyzed against distilled water and lyophilized. For sedimentation analyses, the samples were concentrated by means of an Amicon Diaflo UM2 filter under nitrogen pressure.

SDS-PAGE electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to Laemmli (1970), with the modification that a slab gel was used, containing an acrylamide gradient from 7 - 18 %. Protein samples were dissolved in a solution of 0.0625 M Tris-HCl, pH 7.8, 2 % SDS, 0.1 % DTE, 10 % sucrose and 0.002 % Bromophenol Blue, to a final concentration of 1 mg/ml and incubated at 37^{o} C for 3 hours. Bovine serum albumin, ovalbumin and calf α-crystallin were used as markers.

Isoelectric focusing. Isoelectric focusing in the presence of 6 M urea was performed as described by Van Kleef and Hoenders (1973), using a pH gradient from 5 - 8.

Sedimentation analyses. Sedimentation velocities were determined in a Beckman Spinco E analytical ultracentrifuge, equipped with electronic speed control, and $s_{20,w}$ values were calculated by linear regression analysis.

Determination of the N-terminal amino acid. NH_2-terminal amino acids were identified by the dansyl-chloride method of Gray (1972). Dansyl-amino acids were analyzed by two-dimensional polyamide thin-layer chromatography according to Woods and Wang (1967).

RESULTS.

Gel chromatography. The elution profiles of water-soluble human lens crystallins of various ages are shown in Figs. 1 and 2. The relative amounts are presented in Table I.

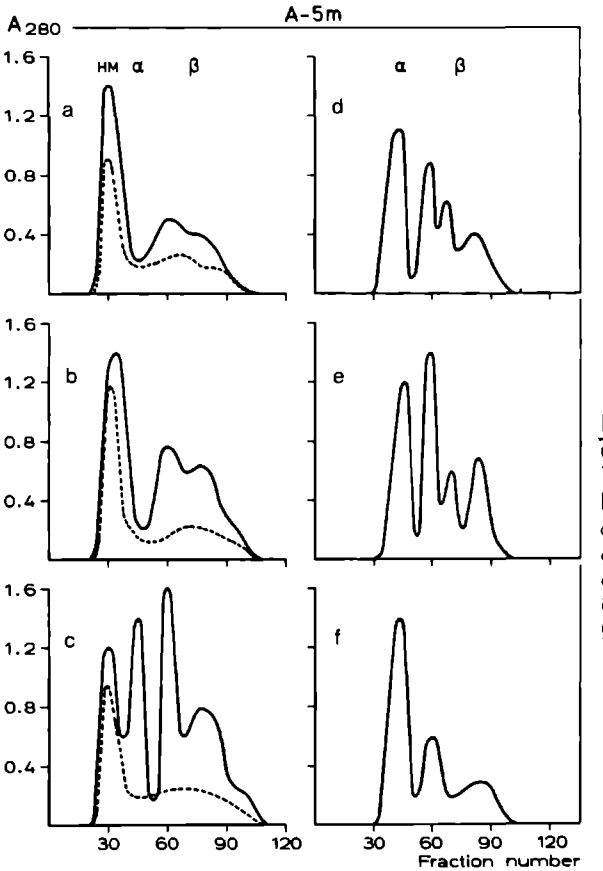

Fig. 1: Elution profiles of water-soluble human lens crystallins on Biogel A-5m. *a)* 76 years *b)* 40 years *c)* 14 years *d)* 3 days *e)* 16-30 weeks of gestation *f)* 8-16 weeks of gestation. (——— = cortex; ------- = nucleus).

Table I Relative percentages of the various crystallin populations at different ages, calculated by measuring the peak areas of the corresponding elution patterns from Figs. 1 and 2.

	HM	α	β	γ
76 year nucleus	28.7	-	50.8	20.5
cortex	23.9	7.6	45.9	22.6
40 year nucleus	28.8	-	47.0	24.3
cortex	17.5	6.5	51.4	24.7
14 year nucleus	19.0	4.9	45.8	30.3
cortex	10.0	16.9	52.7	20.5
3 days	-	21.3	53.7	25.0
16-30 weeks	-	19.3	50.9	29.8
8-16 weeks	-	27.2	53.6	19.1

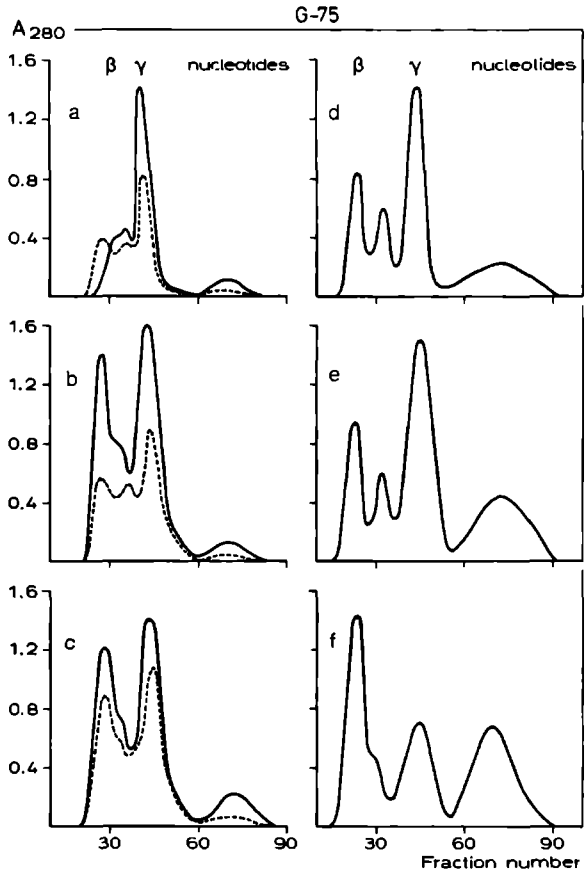

Fig. 2: Elution profiles of the supernatant fraction from human lens crystallins on Sephadex G-75, after centrifugation for 16 h at 78,000 x g; *a)* 76 years *b)* 40 years *c)* 14 years *d)* 3 days *e)* 16-30 weeks of gestation *f)* 8-16 weeks of gestation. (——— = cortex; ------ = nucleus).

In the 14 year-old cortex and in the younger stages the profiles are very sharp, whereas in the 14 year-old nucleus the heterogeneity in the α- and β-crystallin region is already obvious (Fig. 1).

In the 76 and 40 year-old lenses no well-separated low molecular weight α-crystallin peak is observed, neither in the cortex nor in the nucleus (Fig. 1). This peak, still visible in the 14 year-old cortex, appears first in development between the 8th and 16th week of gestation. However, in the 14 year-old nucleus low molecular weight α-crystallin has nearly disappeared. The relative abundance of the latter crystallin class is somewhat higher in the very early fetal stage (Table I).

The relative amount of β-crystallins does not change drastically with the developing lens, whereas the relative amount of γ-crystallin increases in the second trimester of gestation.

SDS-polyacrylamide gel electrophoresis. The electrophoretic patterns for both prenatal and postnatal human crystallins are shown in Fig. 3. In the adult lens (Fig. 3a) α-crystallin is composed of a main band around 20 Kdaltons and minor chains with lower molecular weights. β-Crystallin has main bands around 20 and 25 Kdaltons; some minor chains between 20 and 25 Kdaltons are visible and one just above 25 Kdaltons; in the 43 Kdalton region a diffuse band is seen on the original gel pattern. The γ-crystallin peak was divided into two halves; the first half reveals polypeptides between 19 and 24 Kdaltons, whereas the second half is mainly composed of 19 and 10 Kdalton polypeptides.

The α-crystallin fraction in the 14 year-old lens (Fig. 3b) shows a clear distinction between the A and B chains. In the β-crystallin fraction the 25 Kdalton polypeptide is the major band, but this fraction also contains several polypeptides with higher molecular weights and several chains between 20 and 25 Kdaltons. The weak 43 Kdalton band is less heterogeneous than in the adult lens. γ-Crystallin is mainly composed of chains around 19 Kdaltons in addition to chains between 24 and 20 Kdaltons.

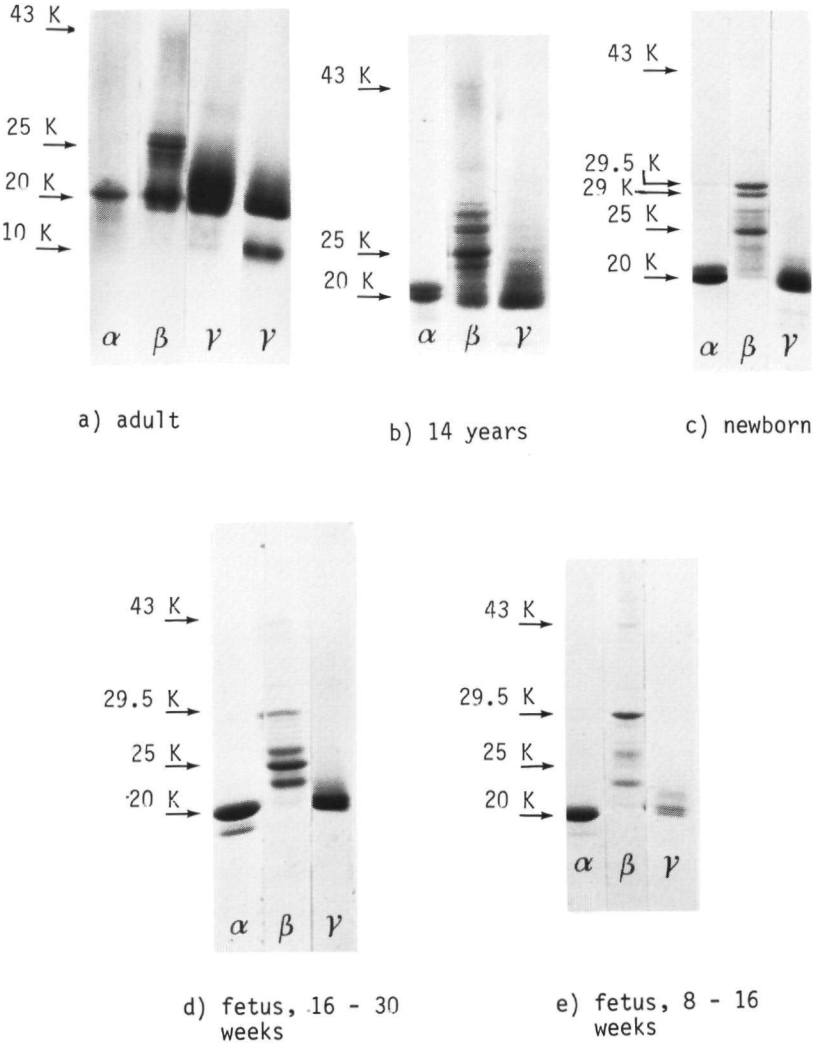

Fig. 3: Sodium dodecyl sulphate gel electrophoresis patterns of various lens crystallins at different ages. The γ-peak from the adult is divided in two halves.

The newborn lens (Fig. 3c) reveals α-crystallin consisting of mainly A chains and to a lesser extent B chains; this is in agreement with findings suggested by the isoelectric focusing pattern (shown later). At this stage, β-crystallin can be characterized by three predominant polypeptide chains: 25, 29 and 29.5 Kdaltons; there also are some smaller chains visible, whereas in the original gel a very sharp, but weak 43 Kdalton band can be detected. γ-Crystallin is less heterogeneous than in the 14 year-old lens and is built up by a higher percentage of 19 Kdalton chains.

α-Crystallin during approximately the second trimester of fetal development (Fig. 3d) contains a distinct 20 Kdalton band and also a sharp band of about 18 Kdaltons. β-Crystallin is composed of the main 25, the 29.5, the 27 and the 23 Kdalton polypeptides. The original gel also shows a sharp but weak 43 Kdalton band. In the γ-crystallin fraction two bands become visible, namely 19 and 20 Kdaltons (which is in agreement with values for calf γ-crystallin as given by Björk, 1964), with a small quantity of chains ranging to 23 Kdaltons.

In the first trimester of fetal development (Fig. 3e) essentially the same situation holds true for α-crystallin, but the 18 Kdalton polypeptide is not clearly visible yet. β-Crystallin consists mainly of a 29.5 Kdalton band; the 25 and 43 Kdalton bands are weak, whereas the 23 and 27 Kdalton polypeptides are clearly visible. γ-Crystallin in this case does not differ from γ-crystallin in the older fetus.

Isoelectric focusing. Isoelectric focusing in the presence of 6 M urea revealed patterns as shown in Fig. 4. Due to lack of material we are not able to present the patterns of newborn and adolescent crystallins.

In the adult α-crystallin has the main polypeptides αA_x, αA_2, αB_x, αB_y and αB_2 (Kramps, de Jong, Hoenders and Wollensak, 1978). Not only in the late, but also in the early stage of fetal development the αA_x, αA_2 and αB_2 chains are very pronounced. In addition we find in both fetal stages a doublet, which is less acidic (pI 6.15) than the αA_2 chain and which is also found in the adult α-crystallin. For the time being we propose the designation αA_y for this band.

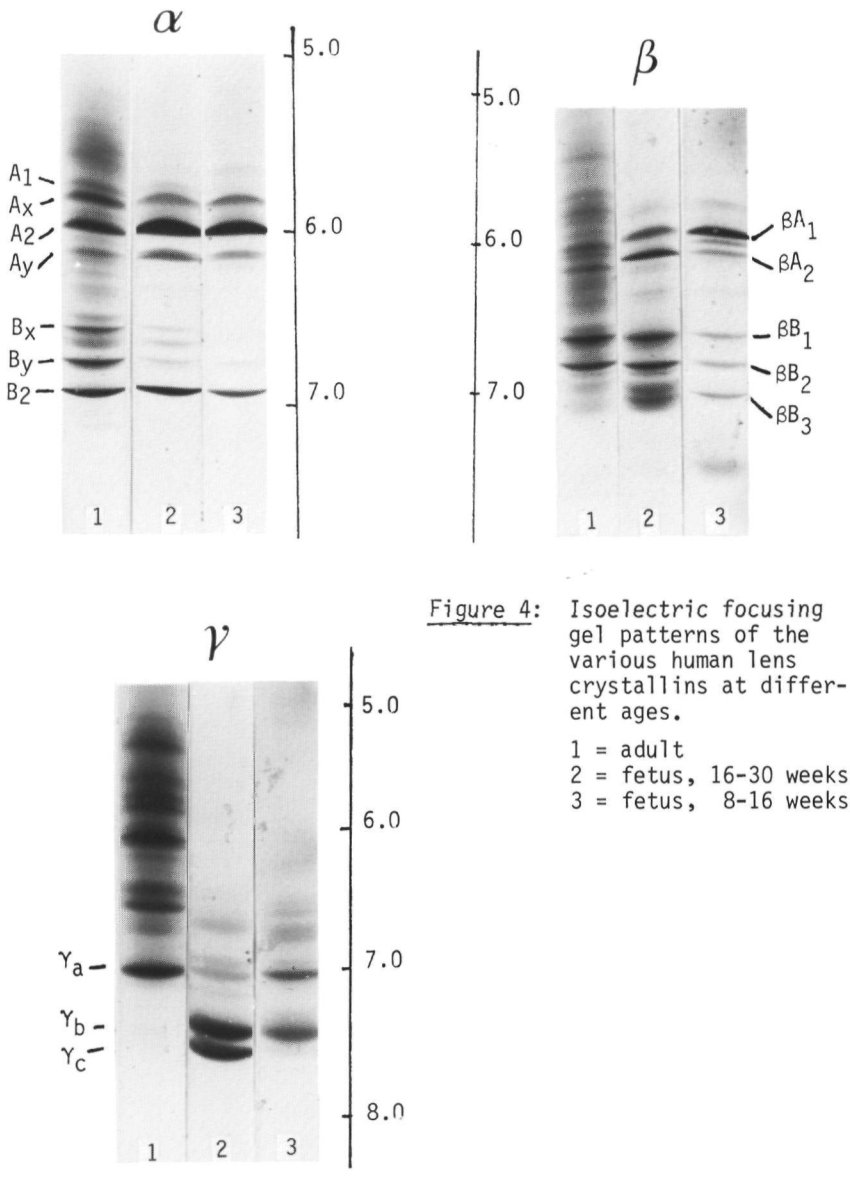

Figure 4: Isoelectric focusing gel patterns of the various human lens crystallins at different ages.

1 = adult
2 = fetus, 16-30 weeks
3 = fetus, 8-16 weeks

αB_x and αB_y appear in later stages, although a very small amount of αB_y is already visible in the first fetal trimester.

In the β-crystallin fraction five bands that are preserved during development and aging can be discerned, namely 2 acidic and 3 basic bands. We propose to designate them βA_1 and βA_2 and βB_1, βB_2 and βB_3, respectively. In the very young fetus there is also a diffuse band at pH 7.5 which has disappeared in the second trimester. As in α-crystallin, many acidic polypeptide chains are seen in the adult.

γ-Crystallin reveals several interesting phenomena:

a) the polypeptide chain at pH 7.0, by which γ-crystallin is distinguishable from the other low molecular weight crystallins (Ringens et al., 1978), is already present in the very early fetal stage and should therefore be typical for the γ-fraction.
b) the band at pH 7.4 increases during development, but disappears completely at some time after birth.
c) between the first and second trimester of fetal development a switch to the production of a more basic chain (pI 7.5) is noted; this chain also disappears after birth.

We have named these chains γ_a, γ_b and γ_c, respectively. In the γ-fraction of the adult lens many acidic polypeptide chains are seen.

Sedimentation analyses. The following $s_{20,w}$ values for adult and fetal crystallins have been found:

	adult	fetal
α	19.5 ± 0.5	17.2 ± 0.2
β_1	7.9 ± 0.2	7.6 ± 0.1
β_2	5.3 ± 0.1	5.1 ± 0.2
β_3	3.6 ± 0.1	3.5 ± 0.1
γ	2.1 ± 0.1	2.2 ± 0.1

With exception that the α-crystallin aggregate seems to become larger with aging, no differences in $s_{20,w}$ values could be found between fetal and adult human lens crystallins. The values for newborn

and 14 year-old lenses were the same as for the adult.

N-terminal amino acid determination. For all ages NH_2-α-amino acid residue determinations were done on the low molecular weight crystallin fraction, eluting in front of the nucleotide peak (see Fig. 2). In all cases amino-terminal glycine was found (in minor amounts in the 40 and 76 year-old lens cortices and nuclei), showing that at all ages at least some γ-crystallin remains.

DISCUSSION.

By comparison of prenatal and adult lens crystallins we observed striking differences in particular as far as subunit composition is concerned (see Figs. 3 and 4). From studies upon the biosynthesis of calf lens crystallins it is known that the primary gene products of α-crystallin are αA_2 and αB_2, whereas αA_1 and αB_1 arise by post-translational deamidation (Strous, Van Westreenen, Van der Logt and Bloemendal, 1974; Delcour and Papaconstantinou, 1972). The same phenomena seem to be the case with human α-crystallin (Spector, Stauffer, Roy, Li and Adams, 1976). Up till now there are no solid data available with regard to post-translational modification of β- and γ-crystallin, with exception of the transition of βB_{1a} into βB_{1b} in calf β_H-crystallin (Vermorken, Herbrink and Bloemendal, 1977). It is however reasonable to assume that what holds true for α-crystallin may also be valid for the other crystallins. In fact our experiments suggest such events for β- and γ-crystallin (Fig. 4), since the shift of the "new" bands is towards the anode, which may reflect their more acidic nature due to deamidation.

The most interesting pattern is that of γ-crystallin, where only 2 - 3 major polypeptides appear after the first 30 weeks of gestation. Although for the time being it cannot be ruled out that one or more γ-genes are switched on during the postnatal period, the diffuse band

pattern of both β- and γ-crystallin is suggestive for post-translational alterations. Moreover, from immunodiffusion tests it is clear that anti-calf-γ-crystallin antiserum does not react with the low molecular weight fraction of the adult human (also observed by Zigler, Horwitz and Kinoshita, 1981), but shows a marked precipitin band with fetal human γ-crystallin (Fig. 5).

From our experiments particularly γ-crystallin turns out to be an extremely useful protein for aging studies.

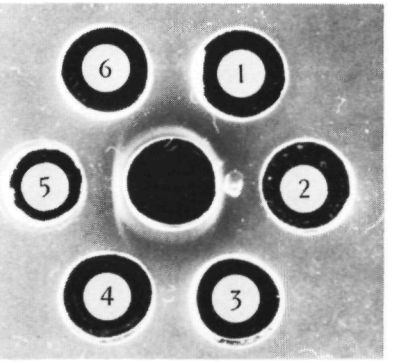

Fig. 5: Immunodiffusion test on human crystallin fractions using anti-calf-γ-crystallin antiserum (center well). Peripheral wells contain 1 mg/ml phosphate buffered saline solutions of the following antigens:
1. fetal α *2.*, *3. and 4.* fetal β
5. fetal γ *6.* adult low molecular weight fraction.

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CHAPTER IV.

BIOSYNTHESIS OF HUMAN LENS PROTEINS IN ORGAN CULTURE.

INTRODUCTION.

Lens protein biosynthesis can be studied under a variety of conditions. Although more reports are available concerning biosynthesis studies in cell-free systems, organ culture has also been described for different species, such as rabbit (Merriam and Kinsey, 1950; Bagchi and Gordon, 1978; Bagchi and Strassman, 1981), calf (Spector and Kinoshita, 1964; Papaconstantinou, 1967; Strous, Van Westreenen, Van der Logt and Bloemendal, 1974), rat (Cohen, Smits and Bloemendal, 1976), pig (Lee, Kauffman and DeVenecia, 1977) and chick (Shinohara and Piatigorsky, 1977; Thomson, Wilkinson, Burns, Truman and Clayton, 1978); however, to our knowledge, only one previous investigation (Spector, Stauffer, Roy, Li and Adams, 1976) deals with the *in vitro* synthesis of human lens proteins.

When studying aging of human crystallins, in particular their role in cataractogenesis, it is important to know which of the polypeptide chains found in aged lens proteins are primary gene products and which arise through post-translational modification. Spector et al. (1976) restricted themselves to the study of α-crystallin. Our investigations on prenatal and postnatal human lenses revealed also interesting changes for the other crystallin populations (Chapters II and III).

In this chapter we describe the biosynthesis of water-soluble proteins in organ-cultured human lenses and their separation by means of gel chromatography. The newly-synthesized crystallins have been characterized by means of sodium dodecyl sulphate gel electrophoresis and isoelectric focusing.

MATERIALS AND METHODS.

Incubation and labeling of fetal lenses. Lenses between 8 and 20 weeks of gestation were incubated in a closed system at 37° C for 20 h (1 lens per tube) under gentle shaking. Each lens was incubated

in 1 ml of modified Minimum Essential Medium (Eagle) containing Earle's salts, 0.2 % sodium bicarbonate, without glutamine and methionine; glutamine (to a final concentration of 2 mM) and (^{35}S)-methionine (25 µCi per lens) were added. The medium was supplemented with 10 % fetal calf serum. After labeling, the lenses were washed with nonradioactive medium, carefully decapsulated and stored at -80° C until analysis.

Separation of water-soluble proteins. Lenses were thawed and gently stirred in 2.0 ml of a fresh buffer containing 20 mM Tris-HCl, 80 mM NaCl, 1 mM EDTA and 10 mM DTE (pH 7.3) at 4° C for 120 min. The sample was rapidly frozen and thawed and then gently stirred once more for 120 min. The resulting homogenate was centrifuged at 10,000 x g for 20 min. The pellet was washed three times with 1 ml of the same buffer and centrifuged. The supernatant fractions were pooled and centrifuged at 78,000 x g at 4° C for 16 h. The 78,000 x g pellet was resuspended in 2.0 ml of the original buffer and fractionated on a Biogel A-5m column (140 x 0.6 cm); the supernatant fraction on a Sephadex G-75 column (100 x 0.6 cm). Radioactivity was determined in a liquid scintillation counter.

SDS-PAGE electrophoresis. Sodium dodecyl sulphate gel electrophoresis was performed as described previously (Ringens, Hoenders and Bloemendal, 1981). Radioactive bands were visualized by scintillation autoradiography (Bonner and Laskey, 1974), in combination with the drying procedure described by Berns and Bloemendal (1974).

Isoelectric focusing. Isoelectric focusing in the presence of 6 M urea was performed as described by Van Kleef and Hoenders (1973) using a pH gradient from 3.5 - 10. The gels were sliced longitudinally, dried as described by Berns and Bloemendal (1974) and autoradiographed with Kodak X-ray film.

In order to improve the separation between γ-crystallin and β-crystallin components, a 78,000 x g centrifugation step for 16 h was applied (Bloemendal and Zweers, 1976). This leaves α-crystallin and the high molecular weight β-components in the pellet. The gel filtration pattern of the proteins from this pellet is shown in Fig. 1;

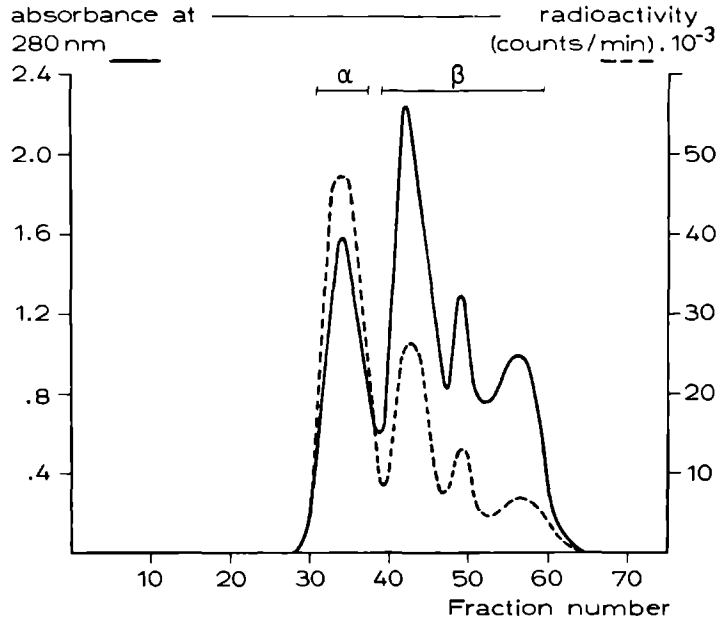

Fig. 1: Biogel A-5m gel filtration patterns of (^{35}S)-methionine labeled proteins, synthesized in organ-cultured fetal human lens cells.

the elution pattern of the supernatant fraction is shown in Fig. 2. It can be seen that a very high specific activity is found under the α-crystallin peak (Fig. 1). Moreover, also under the high molecular weight β-fraction a considerable amount of radioactivity is observed, although lower than in the α-region. This situation is different from calf lens, where β_H is not synthesized *de novo* (Strous et al., 1974).

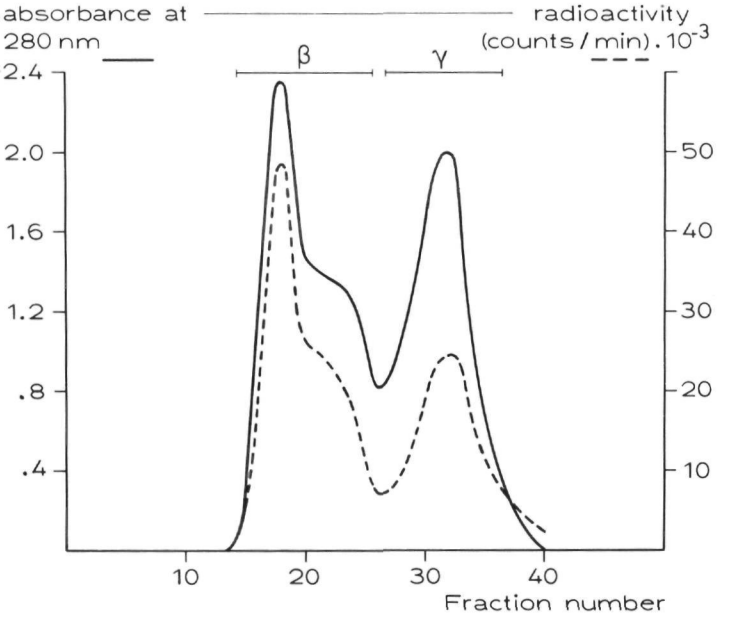

Fig. 2: Sephadex G-75 gel filtration patterns of (^{35}S)-methionine labeled proteins, synthesized in organ-cultured fetal human lens cells.

Fig. 3: SDS-PAGE of fetal human lens crystallins
a) stained pattern b) autoradiograph

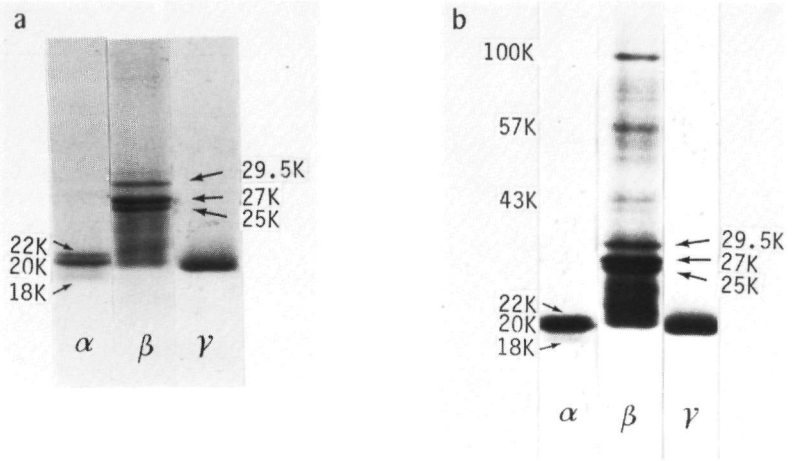

Fig. 3a shows the SDS-gel electrophoresis patterns of fetal human α-, β- and γ-crystallin. The corresponding autoradiographs are presented in Fig. 3b. The stained pattern of α-crystallin reveals the well-known 20 and 22 Kdalton bands, corresponding to the A and B chains, respectively. Furthermore, in the 18 Kdalton region a very weak band is observed. On the autoradiograph bands are seen with the same mobilities; the high amount of A chains is obvious. β-Crystallin comprises major polypeptides of 25, 27 and 29.5 Kdaltons; in addition, on the autoradiograph several chains are seen in the high molecular weight region (43, 57 and approximately 100 Kdaltons). In contrast to various other vertebrate lenses the human β-crystallin polypeptide pattern virtually does not show differences between the high- and low-molecular weight fractions. γ-Crystallin is in both patterns mainly composed of 19-20 Kdalton chains.

The stained isoelectric focusing gel patterns and the corresponding autoradiographs are shown in Figs. 4a and 4b, respectively. α-Crystallin is composed of αA_x, αA_2, the doublet αA_y, αB_x, αB_y and αB_2; radioactivity is detected not only in the αA_2 and αB_2 chains, but also, to a lesser extent, in the αA_x and the αA_y bands. β-Crystallin, consisting chiefly of βA_1, $3A_2$, βB_1, βB_2, βB_3 and some other hitherto undesignated chains, shows radioactivity in each of these bands. Finally γ-crystallin reveals incorporated radioactivity in γ_a, γ_b and γ_c and in an hitherto unidentified acidic γ-chain.

DISCUSSION.

Strous et al. (1974) have shown that in organ-cultured bovine lenses αA_2 and αB_2 had nearly the same specific activity, with no detectable quantity of αA_1 polypeptides and a very low amount of radioactivity in the αB_1 position. Our present results also show radioactivity in αA_2 and αB_2; moreover, radioactivity is seen in the αA_x and in the doublet αA_y. Spector et al. (1976) found only one additional band in the A region with a specific radioactivity of

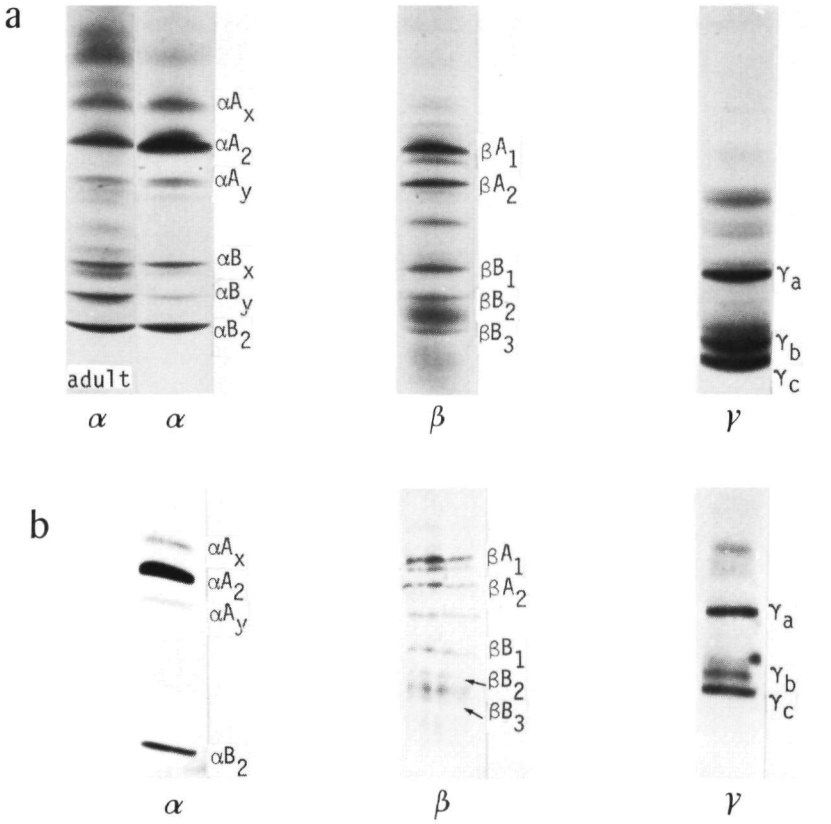

Fig. 4: Isoelectric focusing of fetal human lens crystallins;
a) stained pattern; for comparison adult human α-crystallin is shown.
b) autoradiograph.

approximately 60 % and suggested that this second A chain probably arose by a very rapid transformation process. In this connection the 18 Kdalton chain is of particular interest. This polypeptide also shows radioactivity (see Fig. 3b), indicating that it represents either a primary gene product or a very rapidly formed post-translational

polypeptide. Kramps et al. (1978) found that the αA_x contains a chain with a molecular weight of 18,000 daltons and assumed that this chain is identical with the bovine αA_2^{1-151} (Van Kleef, Nijzink-Maas and Hoenders, 1974). It cannot be excluded that the human prenatal 18 Kdalton chain is also the same constituent as the αA_2^{1-151}; if so, degradation of the αA_2 must have occurred very rapidly (within 20 hours). The 18 Kdalton polypeptide is neither found in very early fetal life nor in the lens of a newborn (Chapter III). Complete degradation may explain the disappearance of this chain. On the other hand, if we assume that αA_2^{1-151} is identical to the 18 Kdalton chain, its post-translational origin is questionable, since it has been shown that the A chain cannot be cleaved between residues 151 and 152 with the aid of endopeptidases (Siezen and Hoenders, 1979).

Apparently also in human lens αA_2 and αB_2 are primary gene products, whereas two additional (acidic) chains are probably post-translational polypeptides, derived from αA_2. The latter assumption is substantiated in particular by the increase of αA_y in the developing lens (Chapter III).

Unlike results obtained in organ-cultured calf lenses where hardly any β_H is synthesized (Strous et al., 1974), the *in vitro* synthesis of high molecular weight β-crystallin in human lens does occur. Moreover, in contrast to calf lenses, newly synthesized 43 and 57 Kdalton polypeptides are observed in the β-fraction (Fig. 3b). Zigler, Horwitz and Kinoshita (1980) have found in adult β-crystallins the 43 Kdalton polypeptide, but the 56 Kdalton chain only in β_1-crystallin; however, we have found both subunits in all fetal β-crystallin samples studied. This finding also sustains our previous observation (Ringens, Hoenders and Bloemendal, 1981) that in contrast to a report by Garner and Spector (1979) the 43 Kdalton polypeptide is present in the prenatal human lens.

Further studies in human lens are required in order to detect possible disorders on the protein biosynthetic level in cataractogenesis.

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CHAPTER V.

THE HUMAN LENS CELL-FREE SYSTEM.

Previous studies in our laboratory showed that the calf lens cell-free system synthesizes and assembles polypeptides that are indistinguishable from isolated native crystallins (Strous, Van Westreenen, Van der Logt and Bloemendal, 1974). Moreover, the complete set of lens membrane-specific components can be synthesized by a reticulocyte lysate supplemented with calf lens polysomes (Vermorken, Hilderink, Van de Ven and Bloemendal, 1975; Ramaekers, Selten-Versteegen, Benedetti, Dunia and Bloemendal, 1980).

It is of interest to know whether the human lens can also provide a viable cell-free system, in particular with the aim in mind to studying the role of a possibly changed synthesis upon aging and the development of cataracts.

This chapter deals with the first attempt to synthesize human lens protein *in vitro* in a heterologous cell-free system.

Four human fetal lenses between 10 and 18 weeks of gestation were used for the isolation of polyribosomes as described for calf by Bloemendal, Schoenmakers, Zweers, Matze and Benedetti (1966). Rabbit reticulocytes were prepared as described by Pelham and Jackson (1976). A 30,000 x g supernatant fraction of the lysed cells was used as cell-free system, and incubations were performed at 30^{0} C for 90 min. The reaction mixture contained per ml: 0.6 ml of reticulocyte cell-free extract, 1 µmol ATP, 0.2 µmol GTP, 50 µg tRNA, 4 µmol dithioerythritol, 10 µmol creatine phosphate, 100 µg creatine phosphokinase, 0.2 µmol spermidine, 100 µmol KCl, 1 µmol magnesiumacetate and 0.1 µmol of 19 amino acids; 100 µl of (^{35}S)-methionine was added as the only labeled amino acid.

The lysate was made mRNA-dependent by preincubation with 10 µg/ml micrococcal nuclease in the presence of 1 mM $CaCl_2$ for 15 min at 20^{0} C as described by Pelham and Jackson (1976). After the incubation 2 mM ethyleneglycol-(2 amino ethylether)N,N'-tetraacetic acid (EGTA)

was added. Polyribosomes were added in a concentration of 0.4 mg/ml (determined spectrophotometrically and assuming an extinction coefficient of 13 at 260 nm).

Analysis of the polypeptides was performed by two-dimensional gel electrophoresis, using isoelectric focusing in the first and sodium dodecyl sulphate polyacrylamide gel electrophoresis in the second dimension (O'Farrell, 1975). Staining and destaining were performed as described by Weber and Osborn (1969). For the detection of the labeled proteins the procedure of Bonner and Laskey (1974), in combination with the drying procedure described by Berns and Bloemendal (1974), was used.

Figures 1 and 2 reveal the two-dimensional gel electrophoretic patterns after staining and autoradiography, respectively.
The following conclusions can be drawn from these two figures:

1) Both crystallin and noncrystallin components can be synthesized in this system (Fig. 2 see for instance spots V, A and T). The αA_2 spot is found on the usual place in the gel (Kibbelaar and Bloemendal, 1975) with a high amount of incorporated activity while virtually no αB_2 is found. This is in concert with earlier findings (chapter III).
   On the autoradiograph radioactivity is clearly visible under the spots migrating with the mobility of actin (A), vimentin (V) and tubulin (T) of calf lens (Ramaekers, 1981).

Fig. 1: Stained two-dimensional gel electrophoretic pattern of the products obtained after translation of human lens polyribosomes; in order to localize the position of newly synthesized lens γ-crystallin (see Fig. 2) calf γ-crystallin has been added.

Fig. 2: Autoradiograph of the two-dimensional gel electrophoretic pattern of the products obtained after translation of human lens polyribosomes. Radioactive markers: (^{14}C)-methylated myosin (200 K daltons), (^{14}C)-methylated phosphorylase b (92 Kd), (^{14}C)-methylated bovine serum albumin (69 Kd), (^{14}C)-methylated carbonic anhydrase (30 Kd) and (^{14}C)-methylated cytochrome c (14.3 Kd).
A = actin; V = vimentin; T = tubulin.

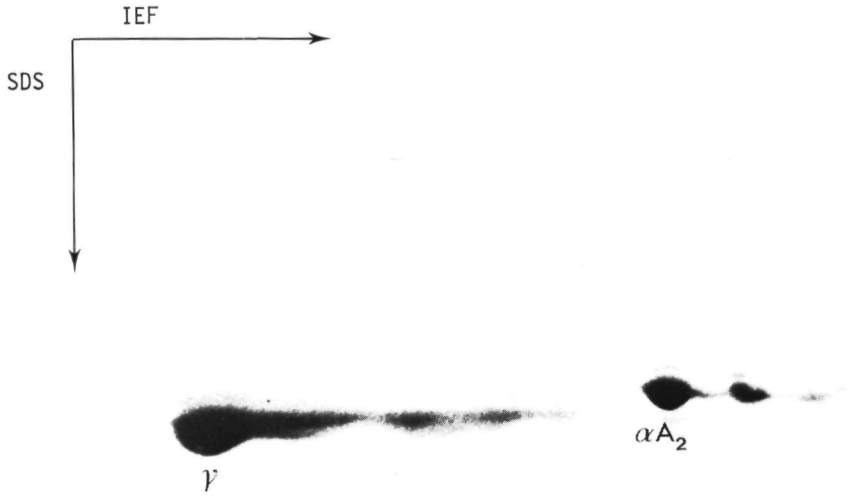

fig.1

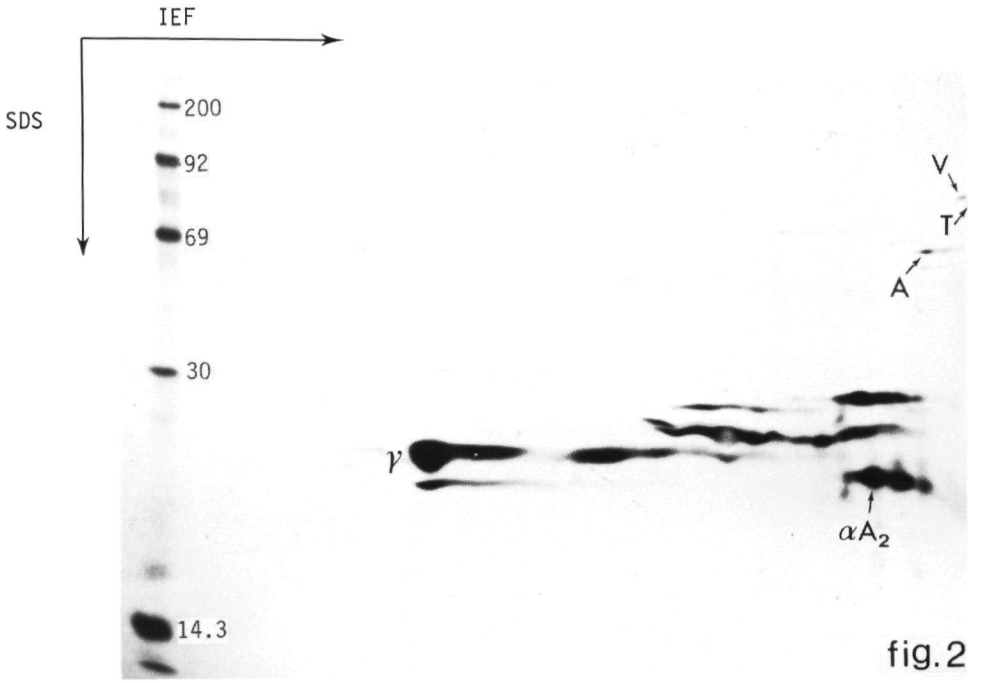

fig.2

2) Calf γ-crystallin (Fig. 1) and the corresponding radioactive spot (Fig. 2) coincide, thus showing that in calf and in man a similar γ-crystallin species is synthesized. The high radioactivity in γ-crystallin also agrees with previous findings (chapter III).
3) In the acidic region of the gel radioactivity can be observed in a polypeptide with an estimated molecular weight of 29.5 Kdaltons. This finding indicates that the subunit βA_1 from the isoelectric focusing pattern (shown elsewhere; chapter III) and the 29.5 Kdalton polypeptide on SDS-gel electrophoresis are identical.
4) A group of proteins with a relatively high amount of incorporated radioactivity can be found in the 25 Kdalton region. Probably these correspond to the 25 Kdalton water-soluble protein, occurring in this stage of development (chapter III) and the water-insoluble component with a molecular weight of 27 Kdaltons (Ringens, Hoenders and Bloemendal, 1981).

The findings presented here show that from fetal human lenses mRNA encoding both lens crystallins and noncrystallin lenticular proteins can be isolated and translated in a heterologous cell-free system. Since we showed that only very few human lenses are required, the technique described appears to be an extremely useful tool for the investigation of aging in the human lens at the translational level.

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CHAPTER VI.

HUMAN LENS EPITHELIUM IN TISSUE CULTURE: BIOCHEMICAL AND MORPHOLOGICAL ASPECTS.

INTRODUCTION.

In cultured calf lens epithelium synthesis of the specific lenticular proteins, the crystallins, rapidly decreases during cell elongation. The presence of one protein of the β-crystallin class has been demonstrated in long-term cultures; however, unlike the situation *in vivo* (Papaconstantinou, 1967), neither α- nor γ-crystallin could be detected upon cell elongation *in vitro* (Van Venrooy, Groeneveld, Bloemendal and Benedetti, 1974). On the other hand an important role has been ascribed to filamentous structures in lens cell elongation *in vitro* (Ramaekers, Hukkelhoven, Groeneveld and Bloemendal, 1979).

Only a few reports are available concerning human lens epithelium in tissue culture. Hamada and Okada (1978) examining 25 day-old monolayers, described the occurrence of piles of elongated cells (so-called "lentoid bodies"), in which they demonstrated the presence of γ-crystallin. These "lentoid bodies" have also been observed by Tassin, Malaise and Courtois (1979) and by Eguchi and Kodama (1979). An ultrastructural comparison of human lens epithelium *in situ* and *in vitro* has been made by Perry, Tassin and Courtois (1979); unfortunately these authors stored their lenses before fixation in a balanced salt solution for 6 - 12 h. Up till now no solid proof has been provided that actually epithelial cells were cultured.

The present chapter deals with:

1) The culturing of human fetal lens epithelium and the ultrastructural confirmation of the epithelial origin of the cells and monolayer.
2) The light microscopical and ultrastructural characteristics of early (1 week) and late (older than 1 month) stage cultures, as well as the comparison of these features to the morphology *in situ* of lens epithelium.
3) The analysis of the proteins synthesized after incubation in a (^{35}S)-methionine containing medium in early and late stage cultures by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

4) An indirect immunofluorescence study on the presence of various filamentous components in the *in vitro* differentiating lens cell.

MATERIALS AND METHODS.

Tissue culture. Human fetal lenses between 8 and 14 weeks of gestation were freshly collected in culture medium. In order to avoid contamination with fibroblasts, the capillary network surrounding the lens at this stage of development, was removed by incubation in a 0.25 % trypsin, Ca^{++}- and Mg^{++}-free Tyrode's solution for 5 min; the lenses were collected in tissue culture medium. The basic medium consisted of 80 % medium 199 (containing 0.5 % lactalbumin hydrolysate) and 20 % fetal bovine serum. For cultivation 4 parts of this medium were mixed with 1 part of the same medium conditioned by human embryonic fibroblasts. The lenses were minced into very small pieces and the material of one pair of lenses was incubated on a coverslip (9 x 50 mm) in a Leighton tube. Occasionally plastic culture dishes were used. For electron microscopy carbon coated coverslips were taken. In rare cases of monolayer formation the cells were subcultured by 0.25 % trypsin in Ca^{++}- and Mg^{++}-free Tyrode's solution.

Electron microscopy. For electron microscopy lens material obtained directly was fixed in diluted Karnovsky fixative (mixture of 2 % paraldehyde and 2 % glutaraldehyde); cell cultures were fixed for 1 - 1.5 h in 2 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2; 415 mosm) at 4^{o} C, rinsed in this buffer and postfixed for 1 h in 1 % osmic acid in the same phosphate buffer. Following dehydration in an ascending series of aqueous ethanol the specimens were transferred via a mixture of propylene oxide and epoxy resin (1:1) into pure Epon 812 as embedding medium. In the case of tissue cultures the coverslips were coated with Epon. After polymerization appropriate areas were selected under a phase contrast microscope. The coverslips were removed using dry ice and the selected areas glued to Epon blanks and sectioned

parallel to the culture surface with glass knives on a Reichert OM U3. The sections were picked up on copper grids, double contrasted with uranyl acetate and lead citrate and examined in a Philips Electron Microscope EM 300.

Labeling of lens cell cultures, preparation of cell lysates and gel electrophoresis. Cultured cells were incubated for 20 h in a methionine-free culture medium, supplemented with (^{35}S)-methionine (5 µCi/ml). After incubation the medium was removed and the cells were rinsed twice with Tyrode solution. Then the cells were harvested with a rubber policeman, washed with Tyrode solution and centrifuged at approximately 2000 x g; this procedure was repeated three times. The cells were then centrifuged at 10,000 x g for 15 min; the pellet was dissolved in 50 µl of SDS-sample solution, frozen and thawed and left at room temperature for 4 h. Sodium dodecyl sulphate gel electrophoresis was performed as described before (Ringens, Hoenders and Bloemendal, 1981); staining and destaining were carried out according to Weber and Osborn (1969). For the detection of the labeled proteins the procedure of Bonner and Laskey (1974), in combination with the drying procedure by Berns and Bloemendal (1974), was used.

Immunofluorescence. Cells grown on coverslips were fixed in -10^{o} C methanol for 6 min. After a brief rinse in phosphate buffered saline (PBS) one of four antisera (anti-actin, anti-prekeratin, anti-vimentin and anti-tubulin) was added as the first antibody for 45 min at 37^{o} C. After washing with PBS, the FITC labeled second antibody was added for another 30 min at 37^{o} C. The coverslips were then mounted in Moviol 4-88 and observed in a Zeiss photomicroscope.

Light microscopy (Fig. 1 A - D; Fig. 2 A - C). During the first 24 hours of culture most of the pieces attached to the glass or plastic surface and the first signs of cell growth were seen at the edges of the fragments (Fig. 1A). During the subsequent days, growth continued and epithelial sheaths surrounding the lens fragments could be observed. The cells first showed bleb-like formations at their periphery (Fig. 1B), revealed later a more regular surface and finally either degenerated or elongated. As a result of this continuous process the epithelium had expanded at the end of the first week, showing simultaneously vacuolar degenerative changes, polygonal cells and some already elongated cells. Thus a dissociation of the original monolayer occurred rapidly, resulting in a trabecular organization of the epithelial cells (Fig. 1C). The process of simultaneous growth and dissociation/destruction took place for several weeks. However, after approximately 3 months *in vitro* the cells were nearly all elongated and signs of cellular aging appeared (Fig. 1D). Especially in the elongated cells the presence of filamentous structures, running parallel with the axis of elongation, was obvious (Fig. 1D, arrow).

In one case the early epithelial sheath around the lens fragments did not dissociate, but formed a confluent monolayer (Fig. 2A) throughout the culture vessel. After 6 weeks *in vitro* subcultivation was possible and the cells were seeded at approximate ratios of 1 : 1.5 and 1 : 2. At the high cell density a new monolayer was formed (Fig. 2B); however, at the lower cell density only local confluent sheaths developed. After 3 months cell elongation, the increased amount of filaments and signs of cellular senescence were also evident in these cultures (Fig. 2C).

Ultrastructural aspects (Fig. 3 A - I).

Lens "in situ" (Fig. 3 A - C). The epithelium is surrounded by a fibrous capsule. The anterior face is covered by the superficial cuboidal epithelial cells. Electron-light and -dense cells can be distinguished (Fig. 3A). A few rough endoplasmatic reticulum (RER)

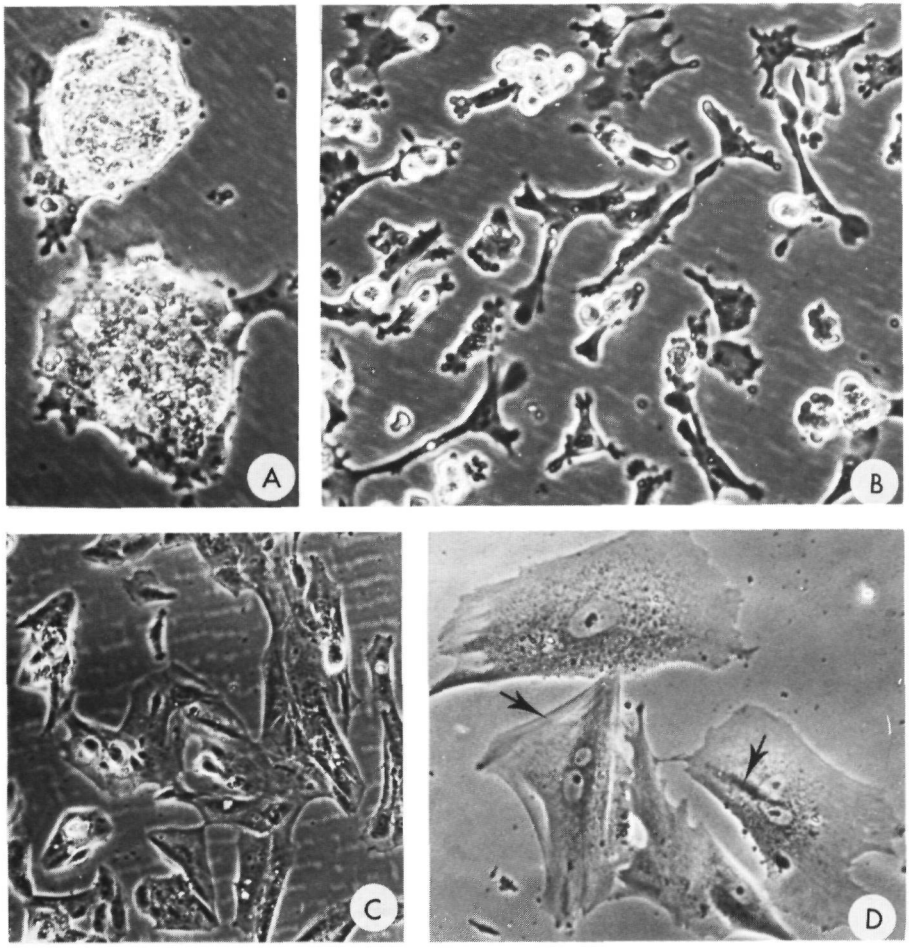


Fig. 1: Phase contrast photographs of human epithelial lens cell cultures.
A) 2 days in culture B) dissociation of cells 5 days in culture C) trabecular organization, 16 days in culture D) elongated and senescent cells; 7th month of culture;
→ = filamentous structures.
(A, B and C: x110; D x220).

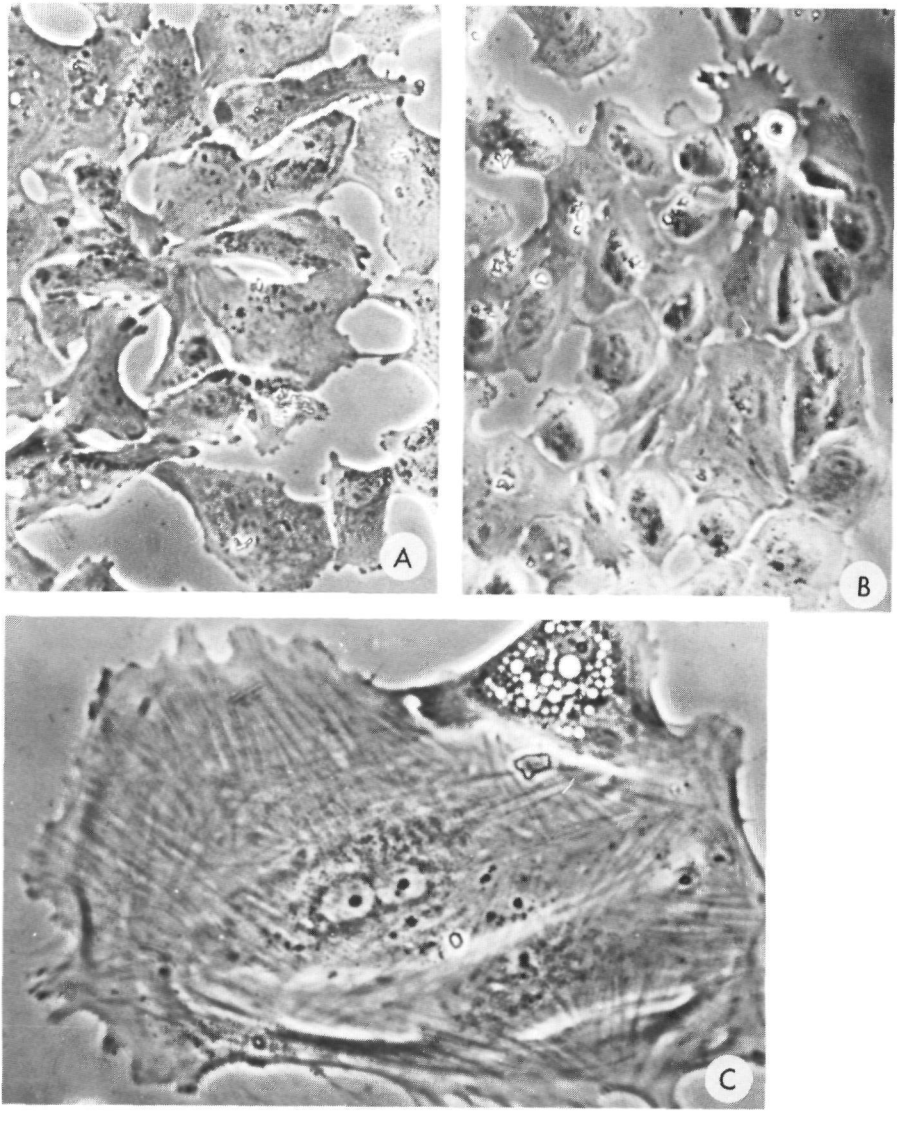


Fig. 2: Phase contrast photographs of epithelial monolayer.
A) primary culture after 1 month
B) first subculture, 5 weeks
C) senescent cells in first subculture, 3½ months.
(A and B x220; C x350).

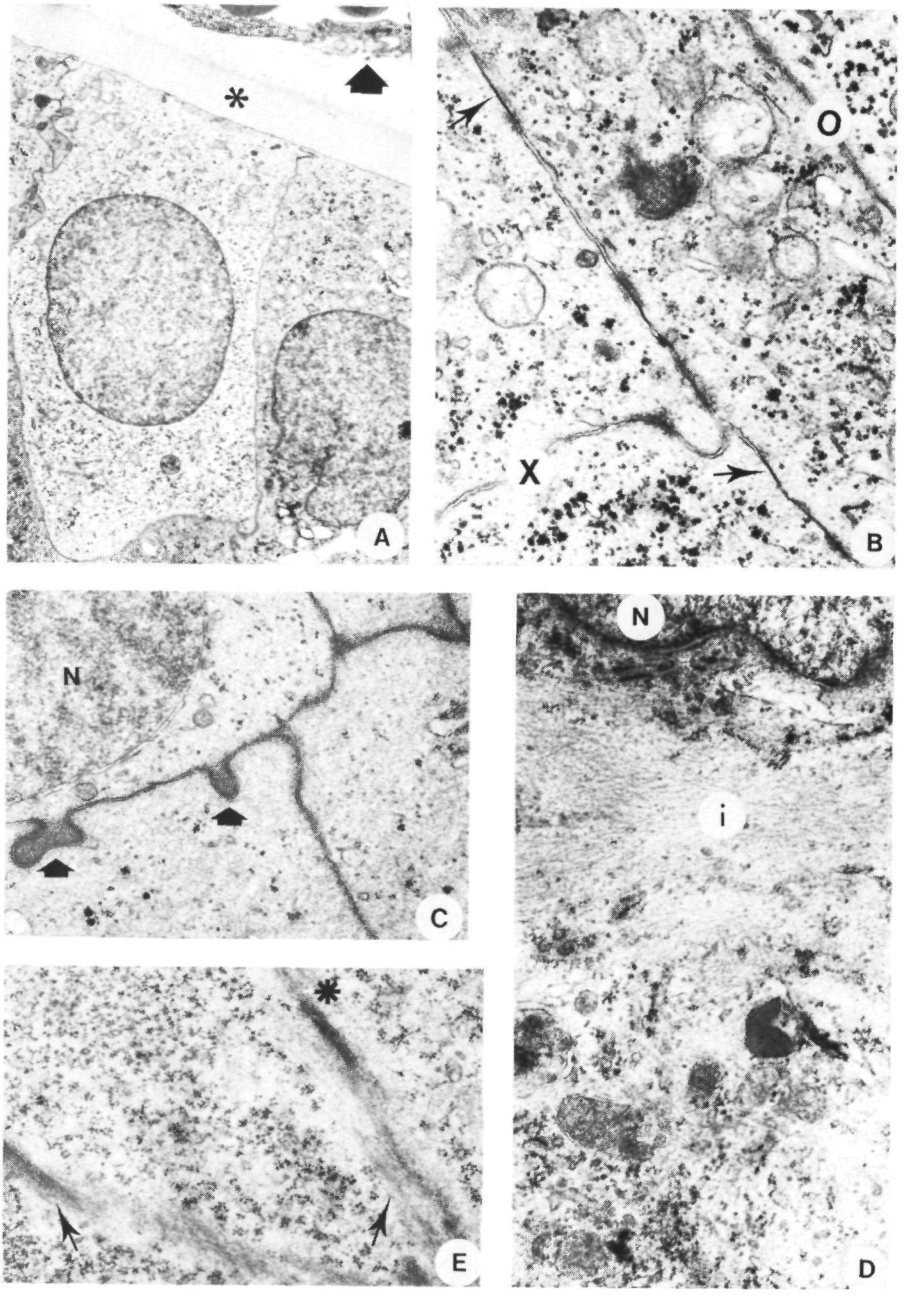
profiles and Golgi areas, as well as a fair amount of juxtanuclear mitochondria are observed. Apart from free ribosomes and polysomes, many glycogen particles (α- and β-form) are striking.

The underlying cortex consists of nuclei-containing lens fibers. The fiber cell nucleus shows a spotty nucleoplasm with a reticular nucleolus. Close to the surface epithelial layer the parallel fibers demonstrate the same distribution of organelles as described above (Fig. 3B). Deeper towards the interior of the lens the cytoplasmic appearance changes gradually with a loss of organelles. It should be emphasized that, though in decreased amount, α- and β-particles and Golgi complexes are still well-recognizable in the most interior cells.

As for the cell membranes, it is shown that the surface epithelial layer is directly adjacent to the multilayered capsular fibrils. Under the plasmalemma thin bundles of microfilaments are localized. The majority of the junctions between the cells are fasciae adherentes; closer to the underlying cortex gap junctions are present, too. The first signs of "ball and socket"-formation are demonstrated in the epithelial layer; this process is more prominent in the interior. Accumulations of microfilaments are present, particularly in these formations (Fig. 3C).

1 day in vitro (Fig. 3 D and E). At the edges of the explanted fragments an epithelioid growth is observed, consisting of large cells with lobulated nuclei and an extended cytoplasm. Apart from the regular organelles, the abundance of polysomes, glycogen particles as well as lysosomal structures should be noted. Both microtubules and intermediate-sized filaments can be discerned (Fig. 3D). The junctions are made up by fascia adherens-like structures, to which many microfilaments attach (Fig. 3E).

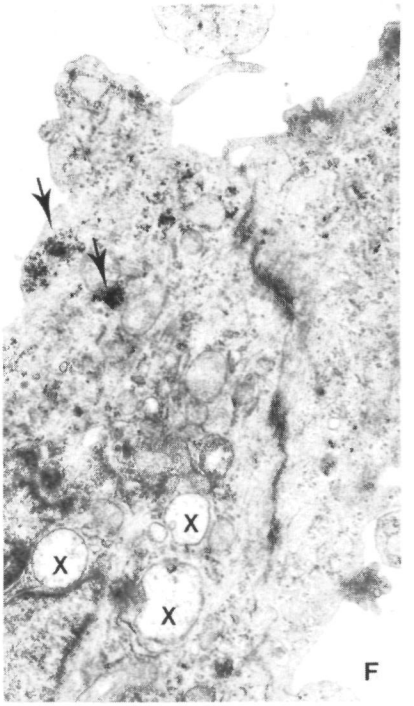
1 month in vitro (Fig. 3 F and G). Different shapes of growing cells are conspicuous in this stage. Nuclei may have capricious forms with distinct nucleoli. The majority of the cells demonstrate an abundance of organelles. The RER is rather dilated and extensively distributed; in between are long, slender mitochondria and an ample amount of free polysomes can be noted. Centrally Golgi areas and some lipid droplets are localized, while lysosomes and glycogen particles are found dispersed as well as in patches throughout the cytoplasm.

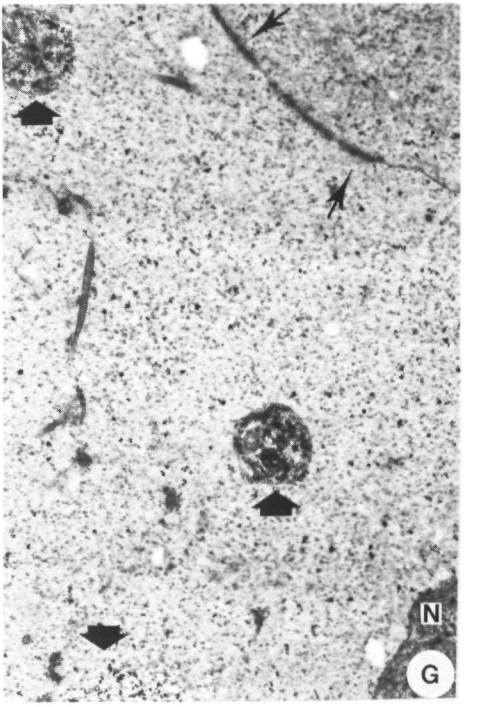

A
O
X
B
N
C
N
i
D
E

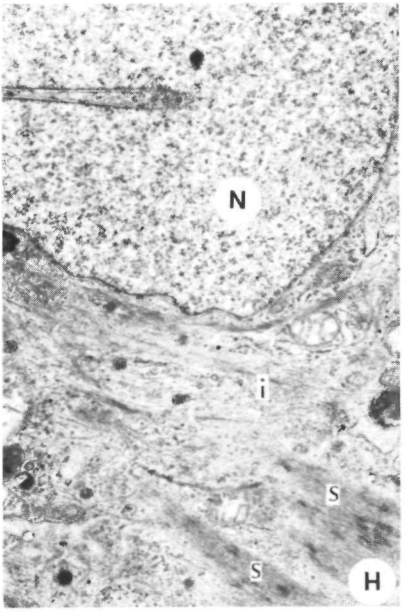
The cytoskeletal feature is as follows: microfilaments as well as intermediate-sized filaments and few microtubules surrounding the nucleus. Towards the periphery the amount of microfilaments increases. The epithelioid nature is obvious as most cells are in contact with each other by their adjacent cell membranes and by fascia adherens-like junctions (Fig. 3F). Additionally, cells with a different (electron-light) cytoplasm are found, too (Fig. 3G); the organelles are decreased in number and are distributed unevenly. Few mitochondria, polysomes, granulated lysosomes and scattered glycogen granules are consistently found; bundles of microfilaments are sometimes observed.

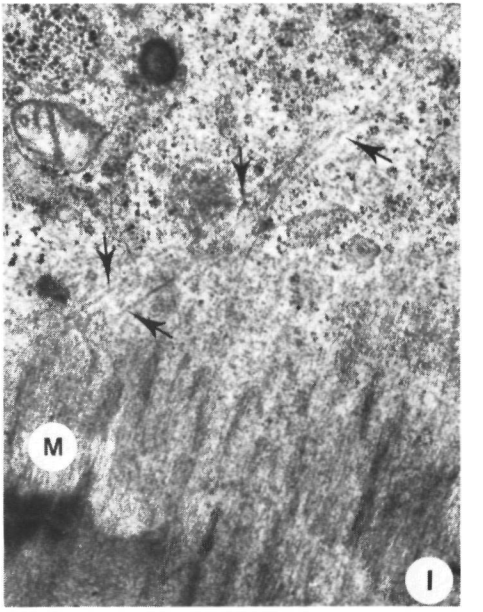
3 months in vitro (Fig. 3 H and I). In this stage the lens cell elongates. The nucleus is bizarre with nucleoli and electron-dense chromatin spots. Centrally few RER and Golgi areas are seen. Mitochondria and lysosomal structures as well as glycogen particles, both scattered and in clusters, are detected. Also a few lipid droplets are found. Most striking, however, is the increase of all types of filamentous structures, especially that of the microfilaments (Fig. 3H). This phenomenon is even more pronounced in the periphery. The same junctions as described above are found in this stage, albeit in greater quantity and longer.(Fig. 3I).

Fig. 3: Electron micrographs of
A) fetal human lens epithelium (HEL) *in situ*, 13 weeks of gestation. Electron-light and -dense cells covered by capsule (*). ➧ = part of a capillary (x3500).
B) HEL *in situ*. X = epithelial cells; note glycogen particles
O = underlying cortical fibers
➔ = gap junctions
(x30,000).
C) HEL *in situ*, interior lens cells; few organelles are present.
N = nucleus
➧ = "ball and socket"-formation
(x21,500).
D) HEL, 1 day *in vitro*.
N = nucleus
i = intermediate-sized filaments
(x15,500).
E) HEL, 1 day *in vitro*. Two neighbouring cells; numerous polysomes and microfilaments (➔);
* = attachment of filaments to junction
(x31,000).


X
X
X
F


N
G


N
i
S
S
H


M
I

Gel electrophoresis (Fig. 4). Fig. 4 shows the sodium dodecyl sulphate gel electrophoretic patterns and the corresponding autoradiographs of cultured lens epithelium after 1 week (Fig. 4A) and 6 weeks (Fig. 4B).

In young cultures (Fig. 4A) a 20 Kdalton chain is present and a fair amount of radioactivity is incorporated in this polypeptide. Moreover, a weak chain is observed at approximately 35 Kdaltons with no distinct corresponding radioactive band. A relatively high amount of radioactivity is seen in the 40-50 Kdalton region and in the higher molecular weight region.

The older cultures (Fig. 4B) reveal a 20 Kdalton polypeptide, with no corresponding band on the autoradiograph. However, two bands with a clearcut radioactivity (at 43 and 57 Kdaltons, respectively) are observed.

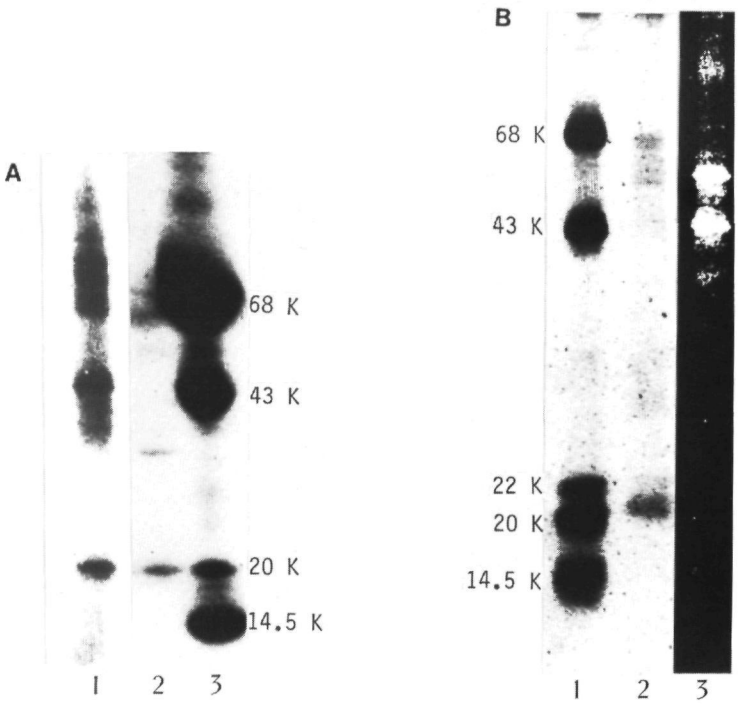
Immunofluorescence (Fig. 5). The results of the indirect immunofluorescence study are presented in Fig. 5. The identity of actin, vimentin and tubulin could be confirmed with highly specific antibodies; no prekeratin was detected.

In young polygonal cells a rather random distribution of the

Fig. 3 (cont.): Electron micrographs of
F) HEL, 1 month *in vitro*. Two adjacent cells with fascia adherens-like junction, to which microfilaments attach.
→ = glycogen
x = lysosomal structures
(x15,500).
G) HEL, 1 month *in vitro*. Electron-light cells. Scattered mitochondria, polysomes and glycogen.
N = nucleus
→ = junctions
➡ = lysosomal structures
(x6500).
H) HEL, 3 months *in vitro*.
N = part of bizarre nucleus
s = bundles of stress fibers
i = intermediate-sized filaments
(x9500).
I) HEL, 3 months *in vitro*.
M = microfilaments attached to fascia adherens-like structure.
→ = long microtubules
Upper left: glycogen and autophagic vacuole with glycogen. (x25,000).

Fig. 4: A) 1. autoradiograph of human epithelial lens cell proteins after SDS gel electrophoresis; 1 week in culture
2. stained pattern of the sample shown in lane 1.
3. markers: bovine serum albumin (68 Kdaltons); ovalbumin (43 Kd); bovine α-crystallin (20 and 22 Kd) and cytochrome c (14.5 Kd).

B) 1. markers
2. stained pattern of human epithelial lens cell proteins after SDS gel electrophoresis; 6 weeks in culture
3. autoradiograph of the pattern shown in lane 2.



filaments is observed. In elongated cells strong arrays of parallel stress fibers are seen in the actin pattern (Fig. 5b), often running across the whole length of the cell in the direction of the elongation. For vimentin and tubulin this organization could not be observed with cell elongation, although for the former some adaptation to the changed cell shape can be seen.

DISCUSSION.

In contrast to calf and chick lens, human lens epithelium has a limited growth capacity, while elongation generally occurs rapidly. According to some recent report the so-called "lentoid bodies" have been described by several investigators (Hamada and Okada, 1978; Eguchi and Kodama, 1979; Tassin, Malaise and Courtois, 1979); these piles of elongated cells are believed to be *in vitro* differentiated lens cells as they are not only elongated but also are reported to contain γ-crystallin. However, it cannot be excluded that the suspected presence of γ-crystallin might be due to crossreactivity of the antiserum.

In the experiments described here the epithelial origin of the cultured cells is established; however, "lentoid bodies" are not seen. This may be due to different culture conditions (Vornhagen and Rink, 1979).It is therefore of interest that Reddan and McGee (1979) also found limited growth capacity of human lens epithelium *in vitro;* moreover, their results also sustain our finding that only some cultures reach confluency and are amenable to subculture.

Van Venrooy et al. (1974) have not been able to show α- or γ-crystallin in established calf lens epithelium *in vitro* and found one β-crystallin polypeptide; Rink and Vornhagen (1979) found γ-crystallin in cultured rat lens epithelium in stage B, but not in later stages of subculturing. In our hands young cultures of human lens epithelium show a reduced synthetic activity compared to the *in situ* situation, whereas in the older cultures no radioactivity

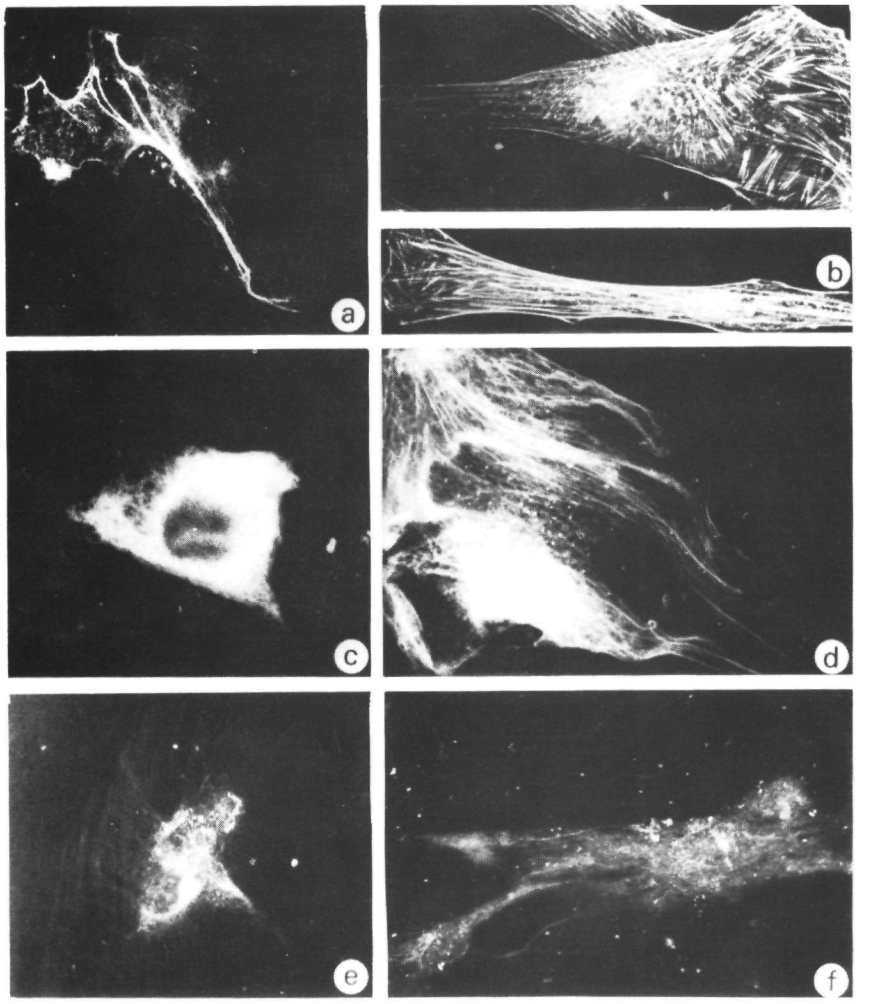


Fig. 5: Immunofluorescence patterns of human epithelial lens cells after 1 week (a, c and e) and 6 weeks (b top and bottom, d and f) *in vitro*.
Incubations were performed with antibodies against actin (a and b, top and bottom), vimentin (c and d) and tubulin (e and f).

at all is incorporated in the crystallin region.

On the other hand, the high amount of radioactivity in the 43 and 57 Kdalton bands in the 6 week-old cultures is striking. These bands comigrate with purified actin and vimentin; additionally, specific antisera directed against these two cytoskeletal proteins react positively upon indirect immunofluorescence; our ultrastructural observations confirm these results. Ramaekers et al. (1979) described the presence of actin bundles (stress fibers) also in elongated calf lens cells *in vitro*, whereas intermediate-sized filaments of the vimentin-type are known to occur in cultured epithelial cells from diverse vertebrates (Franke, Schmid, Winter, Osborn and Weber, 1979). The results presented here suggest that also for human lens epithelium *in vitro* the cytoskeletal components play an important role in the process of maintenance and elongation.

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CHAPTER VII

SUMMARY - SAMENVATTING.

SUMMARY.

In the past 15 years experimental data have accumulated on the molecular structure of the human eye lens. The interest of students of this organ focuses on two main subjects:

1) Aging. Why in particular the eye lens is useful for studying aging processes is described in Chapter I.
   In animal models it has appeared that with differentiation and subsequent aging there is not only a shift in the synthesis of certain lenticular polypeptides, but that these polypeptide chains also undergo post-translational changes, such as deamidation and degradation. These modified products are believed to play a role in processes such as superaggregation and insolubilization. This leads us to the second subject of interest:
2) Cataractogenesis. Again from animal models it became evident that with the development of certain types of cataracts selective shifts in the protein synthesis occur; also aggregation of soluble proteins and subsequent insolubilization takes place when the lens loses its transparency.

It is challenging to draw conclusions from animal models when trying to understand aging processes and the development of senile cataracts in humans.

The bovine lens has been very useful for studying fundamental processes and a comparison with human lenses is justifiable in many respects. However, as shown in Chapter II, the human lens has also some characteristics concerning its protein composition that are different from bovine lenses.

The bulk of human lenses available for biochemical research are extremely old as compared to those from cattle. For that reason one can investigate aged proteins, but hardly any information is available about what proteins are present in the human eye lens before and shortly after birth. This thesis may be a contribution to the elucidation of what happens with the proteins in the aging human lens.

Chapter II gives a survey on the polypeptide patterns of both water-soluble and water-insoluble proteins from a series of lenses of different ages (from fetal stage up to 65 years). It appears that in fact human eye lenses are "aged" by 14 years. In addition it is concluded that the young human lens has some characteristics that are not found in calf lenses.

In Chapter III the distribution and characterization of the water-soluble proteins is given for lenses of different ages. All protein fractions undergo considerable changes not only in the older lens,but also at the prenatal stage. It appears that γ-crystallin in particular is a useful protein for aging studies.

With the exception of α-crystallin, till now no data have been published on the *in vivo* synthesis of human lens proteins. Chapter IV deals with the organ-culture of fetal human lenses. The polypeptide chains described in Chapter III show incorporated radioactivity after a labeling period of 20 hours, thus indicating that they are either primary gene products or rapidly formed post-translational products. The high molecular weight β-crystallin species is also formed in contrast to organ-cultured calf lenses. Moreover, some high molecular weight β-crystallin polypeptides with a high amount of incorporated radioactivity are detected.

Chapter V describes the first attempt to prepare a heterologous cell-free system from human lenses. mRNA encoding both crystallin and noncrystallin lenticular proteins can be isolated and translated. Only very little material is required, which makes this technique suitable for studying synthetic processes in developing, aging and pathological human lens.

Tissue culture of human lens epithelium has been undertaken only recently. Up till now the results have not been impressive, as this material has only a very limited growth capacity *in vitro*. In Chapter VI we describe some biochemical aspects of the aging human lens in culture and relate these to the morphology of the cells. Human lens cells appear to elongate rapidly *in vitro*; after a relatively short period crystallin synthesis ceases and thereafter only cytoskeletal components can be found.

SAMENVATTING.

In de afgelopen 15 jaar is er heel wat onderzoek verricht aan de moleculaire structuur van de ooglens van de mens. De interesse van de lens-onderzoeker richt zich met name op twee onderwerpen:

1) Veroudering. Waarom nu juist de ooglens zo'n geschikt orgaan is voor het bestuderen van verouderingsprocessen wordt beschreven in Hoofdstuk I.
   Uit diermodellen is gebleken dat met de differentiatie en de daaropvolgende veroudering niet alleen een verschuiving plaatsvindt in de synthese van bepaalde lens polypeptiden, maar dat de reeds aanwezige eiwitketens ook post-translationele veranderingen, zoals deamidatie en degradatie, ondergaan. Deze gewijzigde producten worden verondersteld een rol te spelen bij de vorming van grote eiwitaggregaten en bij het onoplosbaar worden van eiwitten; hiermee belanden we bij het tweede onderwerp van interesse:
2) Cataractvorming. Wederom bleek aan de hand van diermodellen dat met het ontstaan van cataract veranderingen optreden in de eiwitsynthese. Bovendien ontstaat er een aggregatie van oplosbare eiwitten, die vervolgens onoplosbaar worden wanneer de lens ondoorzichtig wordt.

Het is verleidelijk om gevolgtrekkingen te maken aan de hand van diermodellen, wanneer we veroudering en het ontstaan van ouderdomsstaar bij de mens proberen te begrijpen.

De lens van de koe is erg nuttig gebleken voor het bestuderen van fundamentele processen en een vergelijk met menselijke lenzen is in veel opzichten gerechtvaardigd. In Hoofdstuk II tonen wij evenwel aan dat de menselijke lens bovendien enige karakteristieken wat betreft zijn eiwitsamenstelling vertoont, die niet kunnen worden vergeleken met de situatie bij de koe.

Om begrijpelijke redenen is de grootste hoeveelheid menselijke lenzen, die ter beschikking komen voor biochemisch onderzoek, uitzonderlijk oud vergeleken met runderogen. Om die reden kan men weliswaar verouderde eiwitten onderzoeken, maar er is nauwelijks enige informatie beschikbaar over de vraag welke eiwitten in de menselijke ooglens vóór

en vlak na de geboorte aanwezig zijn. Dit proefschrift zou een bijdrage kunnen vormen tot de opheldering van de vraag wat er tijdens veroudering gebeurt met de eiwitten, waaruit de menselijke lens is opgebouwd.

Hoofdstuk II geeft een overzicht van de polypeptideketen-samenstelling van water-oplosbare en water-onoplosbare eiwitten van een serie lenzen van verschillende leeftijden (foetaal tot 65 jaar). Het blijkt dat de menselijke ooglens al is "verouderd" op de leeftijd van 14 jaar. Bovendien kan er worden geconcludeerd dat de jonge menselijke lens enkele aspecten vertoont die niet kunnen worden vergeleken met het kalf.

In Hoofdstuk III wordt de eiwitverdeling en de karakterisering gegeven voor lenzen van verschillende leeftijden. Alle eiwitfracties ondergaan veranderingen, niet alleen in de oudere lens, maar ook reeds in het prenatale stadium. Uit deze resultaten wordt geconcludeerd dat met name γ-crystalline een geschikt eiwit is voor de bestudering van veroudering.

Met uitzondering van α-crystalline zijn er tot nu toe geen gegevens gepubliceerd over de *in vitro* synthese van menselijke lens-eiwitten. Hoofdstuk IV behandelt de incubatie van intacte foetale ooglenzen. Alle eiwitketens, beschreven in Hoofdstuk III, vertonen radioactiviteit binnen de periode van labelen (20 uur), hetgeen erop wijst dat ze hetzij primaire gen-producten, hetzij snel-gevormde post-translationele producten zijn. In tegenstelling tot wat er is gevonden bij dit soort experimenten voor het kalf, wordt er in het hier beschreven systeem tóch de groep van hoogmoleculaire β-crystallinen gemaakt. Bovendien vertonen enige hoogmoleculaire β-ketens een hoge mate van geïncorporeerde radioactiviteit.

Hoofdstuk V beschrijft de eerste poging om een heteroloog celvrij systeem te maken uit menselijke ooglenzen. mRNA voor zowel crystallinen als niet-crystallinen kan worden geïsoleerd en getransleerd. Slechts zeer weinig materiaal is nodig, hetgeen deze techniek geschikt maakt om synthese processen te bestuderen in de zich ontwikkelende, ouder wordende en pathologische lens.

Pas onlangs is men begonnen met het kweken van menselijk lens-epitheel. Tot nu toe zijn de resultaten niet erg indrukwekkend geweest, aangezien dit materiaal slechts een zeer beperkte groeicapaciteit

in vitro bezit. In Hoofdstuk VI beschrijven we enige biochemische aspecten van de ouder wordende humane lenskweek en we vergelijken deze bevindingen met de morfologie van de cellen. Het blijkt dat menselijke lensepitheel cellen snel elongeren *in vitro;* na een betrekkelijk korte tijd stopt de crystallinesynthese en kunnen er alleen nog maar cytoskeletaire componenten worden aangetroffen.

DANKWOORD.

Geen lange reeks namen gaat hier de revue passeren. Dat wil overigens niet zeggen dat dit boekje het morfologisch substraat van een ego-trip vormt. Integendeel: een kaft vol met hen, wier hulp onontbeerlijk is gebleken.

Moeilijker valt het om een "tableau de la troupe" van hele afdelingen te krijgen. Vandaar dat ik Prof. Eskes en velen van zijn afdeling (Gynaecologie en Verloskunde, Radboudziekenhuis te Nijmegen) op deze wijze wil danken.

CURRICULUM VITAE.

1 februari 1953 geboren te Nieuwenhagen

mei 1971 eindexamen Gymnasium-β aan het R.K. Gymnasium "Rolduc" te Kerkrade

september 1971 begin van studie geneeskunde, K.U. Nijmegen

maart 1973 - mei 1976 student-assistent afdeling Biochemie, Faculteit der Geneeskunde, K.U. Nijmegen

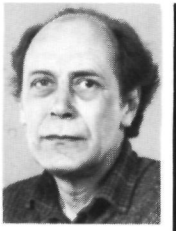
februari 1974 kandidaatsexamen geneeskunde

1974, 1975 en 1976 gedurende een totale tijdsduur van ca. 1 jaar als research-fellow verbonden aan de University of Iowa, USA, Dept. of Physiology (Prof. Dr. G. Edgar Folk). Deze periode werd grotendeels doorgebracht in het Naval Arctic Research Laboratory, Point Barrow, Alaska

januari 1977 doctoraal examen geneeskunde

1 september 1976 - 1 januari 1980 in dienst van de afdeling Biochemie, Faculteit der Geneeskunde, K.U. Nijmegen (Hoofd: Prof. S. Bonting). In deze periode werd het promotie-onderzoek verricht; bovendien werd deelgenomen aan het onderwijs voor eerste- en tweedejaars studenten geneeskunde en tandheelkunde.

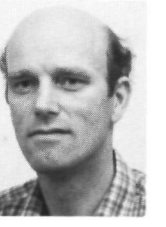
november 1980 semi-artsexamen










STELLINGEN

Nijmegen, 3 september 1981

Peter J. Ringens

I

De conclusie van Manski en Malinowski dat er in de lens complexen van α-, β- en γ-crystallines voorkomen is onjuist, aangezien hun bewijsvoering voor een groot deel steunt op een onjuiste interpretatie van ultrafiltratie experimenten.

Manski, W., Malinowski, K. and Bonitsis, G. (1979). The heterogeneity and structure of complexed alpha-crystallin. Exp. Eye Res. 29, 625-635.

Malinowski, K. and Manski, W. (1980). On the mechanism of alpha-beta-gamma crystallin complex formation. Exp. Eye Res. 30, 527-536.

Malinowski, K. and Manski, W. (1980). Age-related changes in the stability of lens alpha crystallin containing complexes. Exp. Eye Res. 30, 537-543.

II

Wanneer een patient met een niet of onvoldoende verklaarde ketoacidose wordt opgenomen, dient de mogelijkheid van een relatief of absoluut tekort aan thiamine (vitamine B_1) te worden overwogen ("Shoshin"-beri beri).

Majoor, C.L.H. (1978). Alcoholism as a cause of beri-beri heart disease. J. Roy. Coll. Phycns. 12, 143-152.

Majoor, C.L.H. (1978). Het beriberi-hart bij alcoholici. Ned. T. Geneesk. 122, 737-744.

Waarneming J.G.G. Borst, op schrift gesteld door C.L.H. Majoor en L.A. de Vries. Ned. T. Geneesk. 124 (1980) 1411-1416.

III

Bij à terme geboren kinderen met een significante icterus neonatorum, zonder dat er sprake is van bloedgroep-antagonisme, kunnen wisseltransfusies alleen dan door fototherapie worden voorkomen, wanneer daarmee in een vroeg stadium wordt begonnen.

E.J.P. Lommen en J.H.J.M. Meuwissen. Icterus neonatorum in ons land een verwaarlossde bedreiging? Ned. T. Geneesk. 124 (1980) 1685-1688.

IV

Ten onrechte concluderen Epstein et al. dat bij hypermature cataracten phacolytisch glaucoom ontstaat door een lek van grote hoeveelheden hoogmoleculaire eiwitaggregaten vanuit de lens naar het kamervocht.

D.L. Epstein, J.A. Jedziniak and W.M. Grant. Identification of heavy-molecular-weight soluble protein in aqueous humor in human phacolytic glaucoma. Inv. Ophthal. Visual Sci. 17 (1978) 398-402.

V

Bij het instellen van een therapie met benzodiazepines ter behandeling van angsten en spanningen is voorzichtigheid geboden, niet alleen vanwege het ontstaan van lichamelijke en geestelijke afhankelijkheid, maar bovendien en vooral omdat het een maskering van de onderliggende depressie kan betekenen en daarmee een werkelijk causale therapie onmogelijk maakt.

T.T. Oei en A.J.M. Loonen. Benzodiazepines: minder gebruiken is beter gebruiken. T. Alc. Drugs 7 (1981) 22 - 28.

VI

Een hoger verwachtingspatroon, door de omgeving gesteld aan de gehandicapte, zal op zijn minst een socialiserend effect sorteren.

VII

In de lijn van het algemeen streven naar een zo preventief mogelijke gezondheidszorg, waarbij ziekenhuizen een belangrijke rol vervullen, is voor gezondheidscentra de aanwezigheid van slechts één asbak gerechtvaardigd: die bij de ingang.